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**Cue-Induced Uncertainty and Prediction Error: Effects on Nucleus Accumbens
Dopamine and Behavioral Responses to Self-Administered Cocaine and Saline**

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by

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Dedication

The dissertation is dedicated to my parents (Mr. John James D'Souza & Mrs. Monica Scholastica D'Souza) and my wife (Mrs. Preema Lobo D'Souza), for their unconditional love, sacrifice and support during my Ph.D.

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Understanding the process of associative learning between environmental stimuli and cocaine is essential for the prevention of drug-use relapse and long-term treatment of cocaine dependence. Based on contemporary learning theories, empirical studies using natural rewards have shown that cognitive factors, such as uncertainty and prediction errors, play an important role in the process of reward associative learning. Uncertainty is the lack of an accurate predictor for reward while prediction error is defined as the discrepancy between expected and received reward. In this dissertation, we focused on the role of uncertainty and prediction error in cocaine-associative learning. Olfactory and visual cues during self-administration/conditioning sessions were used to induce cocaine-reward expectation and uncertainty in operant trained catheterized Sprague Dawley rats. The influence of cue-induced uncertainty and prediction error on nucleus accumbens dopamine (NAcc DA) following self-administration of cocaine and saline in these conditioned animals was then measured using *in-vivo* microdialysis. Results showed that cocaine-stimulated NAcc DA was enhanced in the presence of cues signaling cocaine

reward uncertainty (Uncertainty) as compared to animals expecting to get cocaine (Certainty). Also omission of expected cocaine reward (Prediction Error) resulted in a significant depression of NAcc DA levels below baseline. Recently diazepam (a positive GABA_A modulator) has been shown to disrupt cocaine-induced LTP and it has been suggested that this disruption can block the acquisition of drug-associated memories. We therefore hypothesized that diazepam-pretreatment during conditioning sessions would disrupt the learned responses to cocaine and saline in the presence of cue-induced uncertainty and prediction error. Our results show that diazepam pretreatment during conditioning sessions, blocked the differential cocaine-stimulated NAcc DA response to cue-induced certainty and uncertainty. Moreover, on omission of expected cocaine reward (Prediction Error) there was no significant depression of NAcc DA below baseline. The findings of this dissertation thus highlight the importance of cognitive factors (uncertainty and prediction errors) in the process of cocaine-associative learning. They also provide a platform to further explore the influence of these factors on other neuroadaptations during cocaine-associative learning, which will help us develop effective behavioral and pharmacological therapies to prevent drug-use relapse.

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Chapter 1: Background & Specific Aims

1-1. Cocaine

Man is always looking for things that can give him pleasure and eternal bliss. In this endeavor to find pleasure, sometimes substances with therapeutic benefit instead become substances of abuse and thus become a danger to the individual and society in general. The story of cocaine is something similar. If I have to describe the story of cocaine in one sentence, I would call it a powerful psychoactive substance with great local anesthetic properties, which slowly over time ended up becoming a substance of abuse [for history of cocaine see (Palfai and Jankiewicz, 1997; Levinthal, 1999)]. There are about 2.3 million chronic cocaine users in United States (see section 1-1-7). This dissertation though is not about how or why cocaine became a substance of abuse. It's about the associative learning between environmental stimuli and cocaine.

Understanding this process of learning is very important, as drug abuse literature suggests that this learning plays an important role in the maintenance and relapse of drug taking habit in humans. This section deals with the source of cocaine, the methods of abuse, its metabolism and excretion, disposition in the brain, its mode of action, short and long term effects of cocaine, current abuse of cocaine in the United States, drug-use relapse and the section finally ends with the relationship between drug-use relapse and associative learning.

1-1-1. Cocaine and Crack

Cocaine is an alkaloid and is found in the leaves of the bush *Erythroxylon coca*, in the amount of about 1% or less, along with many other alkaloids (Lindgren, 1981). As with any other plant, cocaine content varies with growing and harvesting conditions and plant

genetics. The cocaine in the plant exists as a base and is relatively insoluble in water. The alkaloid is extracted and neutralized by an acid to form a stable salt, cocaine hydrochloride or cocaine sulfate, which is readily soluble in water. The other form of cocaine that is extensively smoked is freebase cocaine. Freebase cocaine refers to cocaine that has not been neutralized by an acid. A popular form of freebase cocaine is crack. Crack cocaine is processed with ammonia or sodium bicarbonate (baking soda) and water and heated to remove the hydrochloride. The term 'crack' refers to the crackling sound heard when the mixture is smoked. Legally, cocaine is available as topical upper respiratory tract anesthetic in 1%, 4% and 10% solution (Catterall and Mackie, 2006). However, due to its abuse potential and availability of other local anesthetics, the therapeutic use of cocaine is very limited.

1-1-2. Methods of Abuse

Cocaine is abused in three major ways; it can be smoked (freebase), snorted or injected intravenously (cocaine hydrochloride) (Jones, 1987). Snorting is the process of inhaling cocaine powder through the nose, where it is absorbed into the bloodstream through the nasal tissues. Oral and sublingual intakes are other ways of taking cocaine. Smoking is by far the most efficient and fastest way to deliver the drug to the brain in the most concentrated dose. Smoked cocaine reaches the brain in 6-7 seconds, over the route from the lung to the left heart to brain, whereas an intravenous (IV) injection takes two or three times as long traversing a far longer route (Jones, 1987). Cocaine taken nasally or sublingually begins to reach the brain in 2-3 minutes. Oral intake takes about 8-10 minutes to reach the brain. The rate at which cocaine reaches the brain is important because it determines the onset of the rewarding effects of cocaine. Also, the addictive potential is greater with routes that deliver cocaine rapidly to the brain. Consequently, people who smoke freebase have the highest chance of getting addicted to cocaine.

Choice of route is determined partially by pharmacokinetics and chemistry, but current fashions and fads also influence the mode of administration.

1-1-3. Metabolism and Excretion

Cocaine is extensively metabolized in the blood and liver by enzymes such as liver and plasma carboxylesterase, liver esterase and serum cholinesterase into metabolites benzoylecgonine (BE) (major), ecgonine methyl ester (EME) and ecgonine (Jatlow, 1988; Dean et al., 1991). Only about 1% is excreted unchanged in the liver. Some of the cocaine is converted into norcocaine by hepatic mixed function oxidases. The mean plasma half-life of cocaine following intravenous administration in humans is about 40 minutes and the range is between 19 to 64 minutes (Javaid et al., 1983). Excretion takes place via the urine, which may contain metabolites for 24-36 hour after use. In human subjects, the urinary recovery of BE was (46%) and of EME (41%) of the cocaine dose (Ambre, 1985). Hair samples can also be used to detect use of cocaine.

1-1-4. Brain Cocaine Levels : Correlation with Neurochemical and Behavioral Effects

Cocaine readily crosses the blood-brain barrier (McKim, 1996) and is rapidly distributed into the brain (Pan and Hedaya, 1998). In humans, cocaine binding in the brain is measured by positron emission tomography (PET) and the maximum binding occurs within 4-10 minutes of an intravenous injection of radiolabeled cocaine (Fowler et al., 1989). In animals, brain cocaine concentrations have been measured using *in-vivo* microdialysis. In rats, peak brain concentrations are maximal after 5-8 minutes after an intravenous injection (Pan et al., 1991) and at 20 minutes after an intraperitoneal injection (Bradberry et al., 1993; Javaid and Davis, 1993). Intravenous injection of cocaine in rats has shown that the time course of cocaine concentrations in the brain and the plasma

closely parallel each other, but peak cocaine levels in the brain appear slightly later than that observed in plasma levels (Hedaya and Pan, 1997; Pan and Hedaya, 1998). The concentration of cocaine in the brain following a challenge dose is greater in animals given repeated intraperitoneal cocaine injections as compared to drug naïve animals (Reith et al., 1987; Pettit et al., 1990b). This enhanced increase in cocaine levels, following repeated exposure to cocaine, could be due to a number of mechanisms, such as reduced cocaine metabolism, decreased absorption in adipose tissue and enhanced transfer into the bloodstream (Pettit and Pettit, 1994). Interestingly, the enhanced brain cocaine levels are not seen when cocaine exposure and cocaine challenge are given intravenously (Orona et al., 1994). *In-vivo* microdialysis experiments measuring concentrations of DA and cocaine simultaneously have shown that brain DA and cocaine levels are linearly correlated in both cocaine-naïve animals and cocaine-experienced animals (Nicolaysen et al., 1988; Pettit et al., 1990b). In humans, following an intravenous injection of cocaine, peak plasma concentration was highly correlated with the time course of maximum drug “high” experienced (Javaid et al., 1978).

1-1-5. Cocaine: Mechanism of Action

Cocaine is believed to exert its reinforcing effects by increasing dopamine (DA) in the brain reward pathway (see section 1-3-5 for brain reward pathway) (Wise and Bozarth, 1987; Kuhar et al., 1991; Koob, 1992a). Cocaine binds to the dopamine uptake transporter (DAT), and thus inhibits the uptake of DA from the synapse (Ritz et al., 1987). Dopamine uptake is one of the most important ways of terminating the action of DA in the synapse (Horn, 1990). Thus, the binding of cocaine to DAT increases DA concentration in the synapse. It is hypothesized, that the binding of cocaine to DAT plays an important role in the reinforcing effects of cocaine (Ritz et al., 1987). However, this ‘DAT’ hypothesis. received a critical blow a few years ago with the development of mice with no DAT proteins (DAT knockout mice). Experimental studies suggested that DAT

knockout mice showed both cocaine-induced place preference (CPP) and cocaine self-administration (Rocha et al., 1998). Then again, knocking out DAT protein produces adaptive changes in the DAergic pathways leading to changes in DA synthesis, storage extracellular levels and DA receptor expressions and functions (Giros et al., 1996; Jones et al., 1999). Hence, findings from DAT knockout mice may not reflect the correct picture. The importance of DAT in the reinforcing actions of cocaine was once again highlighted, by showing that cocaine reward is abolished in mice having fully functional DAT protein, which is not sensitive to cocaine binding (Chen et al., 2006).

Cocaine binds to DAT at a site that is distinct from the binding site of DA (Johnson et al., 1992; McElvain and Schenk, 1992). This way, cocaine blocks the uptake of DA in a non-competitive manner and is not itself transported into the cell. The binding of uptake inhibitors to DAT is sodium dependent and it has been shown that cocaine binds to Na⁺ binding site on the DAT (Kennedy and Hanbauer, 1983). Uptake of DA by DAT is both Na⁺ and Cl⁻ dependent. DA transport requires the binding of two Na⁺ ions and one Cl⁻ ion, which are cotransported with DA (Krueger, 1990). Binding of Na⁺ to DAT increases its affinity to DA and promotes its uptake. It is not clear, why other DA uptake inhibitors do not have the same reinforcing effects and abuse potential as cocaine. One point of view is that cocaine binds to sites different from other uptake inhibitors. For example cocaine, mazindol, GBR 12935, GBR 12783 are all DA uptake inhibitors, but have distinct binding sites or domains on the DAT (Berger et al., 1990; Reith et al., 1992). Another possible reason is that cocaine binds to more than one site on the DAT (Madras et al., 1989b). Some studies have shown that cocaine binds to two sites on DAT, while other studies show that cocaine binds to only one site (Madras et al., 1989a; Reith and Selmecki, 1992; Izenwasser et al., 1993; Little et al., 1993; Staley et al., 1994). It has been suggested that DA uptake is regulated by one site on DAT, while behavioral effects are regulated by another site, which is exclusively bound by cocaine. Other uptake inhibitors, which compete with cocaine for binding with DAT may be overlapping at only a subset

of its binding domains (Katz et al., 1997). A third possible explanation is that cocaine does not bind exclusively to DAT, but also binds to SERT (serotonin uptake transporter) and NET (norepinephrine uptake transporter). It is possible that these uptake transporters also play an important role in the reinforcing actions of cocaine. Lastly, a recent report has suggested that cocaine also causes release of DA (Venton et al., 2006).

1-1-6. Short Term and Long Term Effects of Cocaine

Cocaine is a powerful reinforcing drug and people often experience pleasurable high on initial intake. In small doses cocaine can elevate mood and increase energy levels. With high doses cocaine can cause erratic and violent behavior. In addition large doses may also cause tremors, vertigo, muscle twitches, paranoia. Some cocaine users have also reported feelings of restlessness, irritability and anxiety. Cocaine causes an increase in a number of physiological parameters such as heart rate, blood pressure and body temperature (Estroff, 1987; National Institute of Drug Abuse, 2004).

Chronic cocaine use can lead to cocaine dependence. Susceptibility to dependence is determined by a number of individual (e.g. genetic endowment, physiological systems, psychological traits, psychiatric syndromes) and environmental factors (e.g. intrauterine experience, interpersonal factors, societal conditions) (Chilcoat and Johanson, 1998). Chronic use of cocaine also leads to tolerance of the 'high' experienced by people. Tolerance results in abusers increasing their doses to get a greater stimulation and high. Repeated use of large doses of cocaine can cause increasing irritability, restlessness, paranoia, and may lead to full blown paranoid psychosis (Estroff, 1987). Cocaine, especially with chronic use, can also cause medical complications such as disturbances in heart rhythm, heart attacks, respiratory failure, strokes, seizures, abdominal pain and nausea (O'Connor et al., 1992). In addition cocaine abusers, especially those who take

cocaine intravenously, are at more risk than the general population to contract infectious diseases like HIV and viral hepatitis (O'Connor et al., 1992).

1-1-7. Current Abuse of Cocaine in the United States

In 2005, the National Household Survey on Drug Abuse (NHSDA) reported that there are about 2.4 million chronic cocaine users aged 12 or more in the United States (Substance Abuse and Mental Health Services Administration, 2006). Also, an estimated 872,000 initiated cocaine use in 2005. Most of these (62.3%) were 18 years or older when they first used cocaine. Overall, cocaine use was highest in the 18-25 year age group and the average age among the recent initiates aged 12-49 was 19.7 years. In 2004, an estimated 7.8 million responders to a similar survey reported the use of crack of which 467,000 reported current use of crack. It is believed that about 34.2 million Americans aged 12 or older have used cocaine at least once in their lifetime. NHSDA (2004) data also showed an increase in the number of people receiving treatment for cocaine use at a specialty facility from 276,000 in 2003 to 466,000 in 2004. Cocaine related emergencies account for almost half of all the drug related emergencies in the United States.

1-1-8. Drug-use relapse: A major problem with cocaine dependence

Unlike other drugs of abuse, such as ethanol and opioids, physical withdrawal symptoms such as muscle pain, gastrointestinal symptoms, loss of appetite, fever, insomnia from the abrupt cessation of cocaine use are very limited (Gawin, 1991). The major problems faced by patients are psychological symptoms such as intense cravings, depression and lack of motivation (Mendelson and Mello, 1996). Therefore, strategies to counter depression or other psychological problems through medications or psychotherapy form the mainstay of treatment for cocaine dependents. An important issue is to help develop strategies to place the patient back into society, so that he can carry out a more productive

role in society. Following abstinence from cocaine, cocaine dependent subjects are always at risk to fall victim to the vicious cycle of drug abuse, which can occur after a few months, years or sometimes even decades. This return to repeated drug use after a period of abstinence is termed as relapse. Relapse to repeated drug use is an important problem in cocaine dependent subjects (O'Brien et al., 1990) .

1-1-9. Relapse & Associative learning

Relapse, can occur at any time in the life of a person recovering from drug dependence. Although the exact cause of relapse is not known, it is suggested that certain external and internal states can induce strong cravings leading to drug use relapse (Altman et al., 1996). These external and internal states are strongly associated with past drug experience. Examples of external states include environmental situations, paraphernalia (needles, syringes, silver foil, cigarettes, blades) and friends associated with past drug abuse. Internal states such as mental conflict situations, mood states and depression can also lead the individuals to abuse drugs as a crutch to help them overcome these mental situations. Most of our knowledge about the cause of relapse comes from responses of patients who have relapsed. These patients say that exposure to situations or substances associated with past drug abuse leads to strong cravings and urges, which force them to take the drug. In the laboratory, individuals exposed to videos containing objects used for drug abuse, show physiological and neurochemical changes. For example, patients shown cocaine-associated objects report significant cravings (O'Brien et al., 1990; Robbins et al., 1997; Sinha et al., 2000) and show changes in skin temperature, skin conductance and heart rate (Childress et al., 1988; Ehrman et al., 1992). Studies using neuroimaging techniques such as PET (Childress et al., 1999; Volkow et al., 2006; Wong et al., 2006) and fMRI (Risinger et al., 2005; Kosten et al., 2006) have also shown that cue-induced cravings are correlated with activation of brain areas such as dorsal striatum, nucleus accumbens (NAcc) and anterior cingulate cortex. In animals, cues associated with drugs

of abuse readily reinstate drug seeking after extinction of drug seeking behavior in abstinent animals (de Wit and Stewart, 1981; Meil and See, 1996; Fuchs et al., 1998; Grimm et al., 2001). Cues associated with drugs of abuse also retard extinction of drug seeking behavior (Ranaldi and Roberts, 1996; Ciccocioppo et al., 2001; Ciccocioppo et al., 2004). Using second order schedule, studies have shown that animals maintain drug-seeking behavior on contingent presentation of cues (Arroyo et al., 1998; Ito et al., 2000; Ito et al., 2002). Thus, drug-associative learning plays an important role in maintenance and relapse of drug taking behavior and therefore understanding the process of drug-associative learning is essential for the long-term treatment of cocaine dependence.

1-2. Associative Learning

Associative learning between stimuli and reinforcers is not something unique to drugs of abuse. Associative learning occurs with both positive (reward) such as food, water and negative (aversive) reinforcers such as electric shock, painful stimuli etc (Dickinson, 1980). Positive and negative reinforcers have different behavioral effects (Domjan, 1996). For example, positive reinforcers help in acquisition of behavior, maintain learned behaviors and give pleasure on intake. On the other hand, negative reinforcers help in acquisition and maintenance of avoidance behaviors. The environment is full of stimuli, of which not all are important and thus do not require the same attention. The brain has limited resources and hence, it is always essential to identify salient stimuli from the multitude of stimuli in the environment. Salient stimuli are defined as stimuli, which by virtue of their intensity or importance, increase attention, arousal and mobilization of behavioral resources, thus contributing to action (Redgrave et al., 1999; Horvitz, 2000). Learning to associate environmental stimuli with rewards or aversive stimuli, is critical for survival. For example, stimuli associated with food help animals to prepare and move rapidly to acquire food, especially in contexts where food is scarce, and competing predators exist in the environment. Similarly, stimuli associated with aversive outcomes

help animals to take protective action, and thus protect them from harm. In our everyday life, we use associative learning to get prepared and take action based on our past experience with stimuli. For example, while traveling on the subway, the sound of an approaching train alerts us to stand at an appropriate distance to avoid being hit by the approaching train. Similarly, while selecting fruits in the grocery store we pay a lot of attention to the appearance of the fruit, to determine which fruit will taste the best. Thus our selection of the fruit has been based on our past experience. Associative learning in addition to being critical for survival, also plays an important role in the process of decision-making. Thus understanding associative learning will help us to decipher the complex process of decision making, which in turn may be useful in developing artificial intelligence.

1-2-1. Role of Prediction Errors and Uncertainty

According to current learning theories (Rescorla and Wagner, 1972; Wagner, 1981; Pearce, 1987; Rescorla, 2006a) the discrepancy between expectation of reward and outcome received, plays an important role in associative learning. This discrepancy is termed as prediction error. Expectations are built through experience and signals or cues in the environment. For example, the picture of a Coke can on a vending machine indicates that on pressing the button, one will receive a can of Coke. Once you have learned that association, the picture of a Coke can on any vending machine will build an expectation of receiving Coke on pressing the appropriate button. Now, a prediction error will occur if on putting the money and pressing the appropriate button due to some malfunction of the machine, one does not end up with a can of Coke. Thus, there is discrepancy between expectation and outcome. This type of prediction error is called ‘negative prediction error’, because there was an expectation to get a can of Coke, but nothing was obtained. On the other hand, if on pressing the button two cans of Coke were given instead of one, there is a discrepancy between expectation and outcome. In this

case since one gets more than one expected, this type of prediction error is called 'positive prediction error'. Prediction errors can modify behavior. For example, if one experiences negative prediction error on frequent basis, one will try and avoid that machine, unless of course one likes losing money in the vending machine. On the other hand if one keeps getting two cans of Coke, one might try and keep coming to the same vending machine. One may also choose to call the company and ask to get the machine fixed. In any scenario, prediction errors have modified behavior. Greater the discrepancy, greater is the learning. However, if the vending machine works perfectly and there is no discrepancy between expectation and outcome, the rate of learning plateaus. In this dissertation, expectation of receiving cocaine reward was built through conditioning by consistently associating one set of olfactory and visual cues with cocaine and another distinct set of cues with non-reward (saline). After conditioning, a negative prediction error was induced on the test day, by letting animals self-administer saline (non-reward) in the presence of cues consistently associated with cocaine (reward). On the other hand a positive prediction error was induced, by letting animals self-administer cocaine (reward) in the presence of cues consistently associated with saline (non-reward).

Another school of thought suggests that environment induced uncertainty plays an important role in learning (Pearce and Hall, 1980). Uncertainty is the lack of an accurate predictor for the occurrence of a reinforcer. The lack of an accurate predictor, however does not mean an absence of a predictor. According to this theory, uncertainty enhances attention to the environment, and this enhanced attention helps in the process of associative learning. To understand this theory further let's again take the example of the vending machine. If this time, there are no signs or pictures on a vending machine and it is mixed with different drinks such as Coke, Pepsi, Sprite etc. Pushing a random button may or may not result in getting one's desired drink. The lack of a predictor creates uncertainty with respect to the outcome. As a result, greater attention will be devoted to the machine in search of additional clues. Thus, the lack of an accurate predictor has

heightened one's attention. Uncertainty is a part of our everyday life and there are several occasions daily where outcomes are uncertain. For example, when one buys a watermelon or bunch of grapes, one is not really sure how they will turn out, there is some degree of uncertainty with respect to the outcome. One is certainly happy if they end up being sweet. People react to uncertainty in different ways: some people take impulsive decisions, some may take unnecessary risk, some take cool calculated decisions while still other may not take any action. Retrospective surveys in people suffering from drug-dependence have shown, that impulsivity and high risk taking behavior are common personality traits in these people (Dawe and Loxton, 2004). Thus, it is possible that initiation of drug taking may be an impulsive decision, taken when faced with uncertain outcomes. It is possible, that many people experience drugs of abuse under conditions of uncertainty and hence it is important to evaluate the impact of uncertainty on neurochemical and behavioral response to drug-reward. In this dissertation, uncertainty with respect to cocaine-reward occurrence was induced, by associating cues equally with both cocaine and non-reward (saline) during conditioning. The animals thus lacked an accurate predictor of cocaine-reward occurrence.

1-2-2. Drug-Associative Conditioning Paradigms

Several different conditioning paradigms exist to assess associative learning. The focus in this dissertation, will be limited to conditioning paradigms commonly used in studying drug-associative learning. The process of conditioning involves pairing of two stimuli, one of which is called the neutral stimulus. The neutral stimulus does not by itself elicit any response. However, the second stimulus usually a reinforcer, produces a response called the unconditioned response. Repeated and temporally contingent pairing of neutral stimulus with the reinforcer (unconditioned stimulus) leads to a learned association between the stimulus and the reinforcer, which is referred to as conditioning. Following consistent pairings with the unconditioned stimuli, the previously neutral stimulus is now

capable of eliciting behavioral or neurochemical responses, and is now called a conditioned stimulus. Studies have shown that conditioning depends on several factors, such as nature of the stimuli, information provided by the neutral stimuli about occurrence of the reinforcer, reinforcer used and number of trials (For a more detailed review on conditioning see (Rescorla, 1988). Currently, scientists consider conditioning to be a process that involves the learning of relations between events.

Conditioning can be broadly classified as Pavlovian conditioning or Instrumental conditioning. Pavlovian conditioning, is a type of conditioning in which the subject is passively exposed to both stimuli and does not have to perform any action. Conditioned place preference (CPP) is an example of Pavlovian conditioning and is commonly used to study behavioral and neural correlates of drug-associative learning (Tzschentke, 1998; Duvauchelle et al., 2000b). This paradigm consists of a box with two chambers providing two distinct environments. One of the chambers is associated with repeated cocaine (drug) injections and the other chamber is associated with repeated saline (control) injections. On the test day animals have access to both, drug and saline associated chambers. If the drug is rewarding animals will occupy the drug-associated chamber for a longer duration as compared to the saline-associated chamber. In this model, one needs to ensure that the animals do not have a bias for any one side more than other, prior to drug-conditioning trials. CPP is a simple and reliable way to study drug-associative learning (Spyraki et al., 1982; Bell et al., 1997; Duvauchelle et al., 2000a). However, a major disadvantage of the model is that the drug is experimenter administered and not under the control of the animal. This aspect is important in drug-abuse studies, because differences in neurochemical responses have been shown to occur between self-administration and experimenter-dependent administration of drugs of abuse. For example, Hemby et al. (1997) showed that nucleus accumbens (NAcc) dopamine (DA) response was greater, when animals self-administered cocaine, as compared to animals receiving yoked administration of the same dose of cocaine (Hemby et al., 1997). Similarly, repetitive

self-administration of cocaine results in increased concentration of acetylcholine in the NAcc shell, as compared to yoked administration of the same dose of cocaine (Mark et al., 1999). Also, cocaine-induced glutamate release in NAcc core is enhanced after extinction of self-administration, but not from withdrawal of passive administration of cocaine (McFarland et al., 2003). Thus, self-administration plays an important role in drug addiction research.

The other major type of conditioning is instrumental conditioning. In this type of conditioning the animal has to perform an operant response (R) and learn the relationship between the response and reward (S) following that action (Domjan, 1996). This type of conditioning can be further modified by introducing cues (S1) prior to the animal making an operant response (R), so that the animal can learn the relationship between cues (S1), operant response (R) and reinforcer (S2). In other words, the animals have to learn S1-R-S2 relationship. This type of operant conditioning was used in the experiments presented in this dissertation. The advantage of instrumental conditioning is that the animals voluntarily take the drug, which has greater clinical relevance. Self-administration of the drug also addresses the self- vs. experimenter-administered problem mentioned earlier. Another advantage is that animals develop expectations of forthcoming reward based on the information provided by the cues prior to making an operant response.

Although, the main conditioning used in this dissertation was instrumental, the precise paradigm involved some aspects of CPP (Ikegami and Duvauchelle, 2004a). For example, the animals were conditioned with 16 days of alternating cocaine and saline self-administration sessions. The alternating day cocaine and saline exposure is often used in the conditioned place preference model. This type of paradigm allowed for specific cues to be associated with cocaine and saline and animals developed expectations of reward and non-reward. In order to avoid confusion in terminology, the self-administration/ conditioning sessions will henceforth be referred to as 'cue-associative

training' in this dissertation. Intravenous self-administration of cocaine is easily established and can be maintained over long periods of time (Emmett-Oglesby et al., 1993; Koeltzow and Vezina, 2005). A dose of 0.5 mg/kg/infusion of cocaine was used during cue-associative training sessions and cocaine is readily self-administered by rats at this dose (Pettit and Justice, 1991). The dose also falls within the intravenous dose range commonly abused by humans, which is between 0.35 mg/kg and 0.71 mg/kg (25-50 mg/day) (Volkow et al., 1997). A combination of olfactory and visual cues were used during cue-associative training, because it has been shown that a compound stimulus helps discriminative learning and enhances responses during operant conditioning (Emurian and Weiss, 1972; Weiss, 1972; Wiltz, 1974). Olfactory cues are particularly effective in rats, because rats have a complex olfactory epithelium, which is densely packed with olfactory sensory neurons. Moreover, the olfactory bulb in rats is relatively large as compared to the rest of the forebrain, and projects to important regions such as the prefrontal cortex, hippocampus, entorhinal cortex, amygdala and the hypothalamus (Cain, 1974; Slotnick, 2001). These regions play an important role in emotion, learning and memory. The sense of smell is very important, and guides every aspect of a rat's life. After this brief review of conditioning models for drugs of abuse, a review of dopamine, a key neural substrate that plays an important role in associative learning and cocaine reinforcement (Schultz, 1998; Di Chiara, 1999) will follow.

1-3. Dopamine

Dopamine (DA) is a catecholamine and was described as a neurotransmitter by Arvid Carlsson in the 1950's. Over the last 50 years, DA has been shown to play an important role in neuropsychiatric disorders (e.g. Attention deficit hyperactive disorder (ADHD), Schizophrenia, Obsessive-compulsive disorders), neurological disorders (e.g. Parkinsonism), drug and natural reward reinforcement and learning and memory (Wise, 2004; Iversen and Iversen, 2007). In this dissertation, we look at the role of DA in

learning and memory associated with drug reward. In the following sections, synthesis, storage, metabolism of DA, dopaminergic receptors and their signal transduction pathways will be discussed. In addition, the role of DA in molecular and synaptic mechanisms of learning and memory and dopaminergic pathways in the brain will also be discussed.

1-3-1. Biosynthesis, storage and metabolism

DA is synthesized from tyrosine (amino acid) and its rate-limiting step is the conversion of L-tyrosine to L-DOPA by the enzyme tyrosine hydroxylase (TH) (Cooper et al., 2003). DOPA is subsequently converted to DA by L-aromatic amino acid decarboxylase at rates so rapid that DOPA levels in the brain are negligible under normal conditions. The levels of endogenous DOPA are low and therefore synthesis of DA can be greatly increased by providing L-DOPA exogenously. The activity of tyrosine hydroxylase is regulated by DA, presynaptic dopaminergic autoreceptors, enzyme cofactors and the activity of dopaminergic neurons (Cooper et al., 2003). The newly formed DA is stored in vesicles and released into the synaptic cleft. The release of DA into the synaptic cleft is calcium dependent. The action of DA is mainly terminated by its uptake from the synaptic cleft by the dopamine uptake transporter (DAT) (Cooper et al., 2003). The DA that is taken back into the presynaptic neuron is converted into dihydroxyphenylacetic acid (DOPAC) by intraneuronal monoamine oxidase (MAO). Some DA may be metabolized by catechol-O-methyltransferase (COMT) in the synaptic cleft (Cooper et al., 2003). In the rat brain, DOPAC and HVA (homovanillic acid) are present in sulfate conjugated as well as free form. HVA is the major metabolite in humans and primates and only a small amount of it is found in the free form.

1-3-2. Receptors and Signal Transduction Systems

The released DA acts on receptors present in synaptic and extrasynaptic spaces. The dopaminergic receptors are G-protein coupled receptors and can be classified into two broad categories: D1-like family of receptors and the D2-like family of receptors (Missale et al., 1998). The D1-like family of receptors consists of the D1 and D5 receptors, while D2, D3 and D4 receptors constitute the D2-like family of receptors. D1 like receptors couple to stimulatory G proteins ($G_{s\alpha/Olf}$) that activate adenylate cyclase, while D2-like receptors inhibit adenylate cyclase (Missale et al., 1998). Activation of adenylate cyclase promotes the formation of 3',5'-cyclic adenosine monophosphate (cAMP) formation through ATP hydrolysis. cAMP binds to the regulatory subunits of cAMP dependent protein kinase (PKA) and thus activates the catalytic subunits of PKA (Mellon et al., 1989). Additionally, both families of DA receptors also interact with other signal transduction pathways. For example, activation of D1-like receptors reportedly activates phospholipase C (PLC), thus stimulating phosphatidylinositol hydrolysis and the mobilization of intracellular calcium stores (Liu et al., 1992; Undie et al., 1994). D2 receptor stimulation enhances potassium conductance through inward rectifying channels (Jackson and Westlind-Danielsson, 1994) and attenuates calcium influx via voltage-gated channels (Missale et al., 1998).

DA receptors can also be classified based on their location on the neurons. Autoreceptors are present presynaptically on dopaminergic terminals. Postsynaptic receptors are present on postsynaptic neurons within the synapse. Juxtasympaptic receptors are present just outside the dopaminergic synapse and heteroreceptors are present presynaptically on terminals of neurons releasing neurotransmitters other than DA. D2 receptor plays an important role as an autoreceptor, regulating the release of DA from the terminal and soma (Cooper et al., 2003). The affinity of DA for the two families of dopaminergic receptors (i.e. D1-like and D2-like) is also different. Both D1 and D2 receptors can exist

in high affinity and low affinity states. The majority (80%) of D1 receptors are in low affinity state (2-4 μM), while 20% are in the high affinity state (9-74 μM) (Richfield et al., 1989). On the other hand, upto 10% of D2 receptors exist in the low affinity state, while 80-90% exist in the high affinity state (Richfield et al., 1989). In general, D1 have 100 times lower affinity than D2 receptors. The activation of DA receptors can depend on the amount of DA that is released. If a large amount of DA is released in the synapse, then it is likely to activate the low affinity D1 receptors. Similarly, if the amount of released DA is small, then it is likely to activate high affinity D2 receptors.

1-3-3. Molecular Substrates of Dopamine-Dependent Learning

If DA plays an important role in learning and memory, then activation of dopaminergic receptors should lead to molecular changes that are critical for learning and memory. Cocaine increases DA in brain reward pathways and repeated administration of cocaine leads to adaptations in DA receptor signaling (Anderson and Pierce, 2005). One consequence of that adaptation is the upregulation of D1-cAMP-PKA pathway (Terwilliger et al., 1991). The upregulation of this pathway leads to increased concentrations of adenylyl cyclase, PKA and other components of this signaling pathway (Nestler and Aghajanian, 1997). The up-regulation of D1-cAMP pathway may lead to supersensitivity of D1 receptors following repeated cocaine administration (Henry and White, 1995; Nestler, 2001). Although the mechanism of this upregulation is not known, it is reported that at least in the prefrontal neurons, this up-regulation can take place through activator of G protein signaling-3 (AGS3) (Kalivas et al., 2005). AGS3 is a negative regulator of G_i and hence of D2 signalling. Therefore, up-regulation of AGS3, can lead to shift of dopaminergic signaling via the D1-cAMP pathway. The question is however, what has this shift got to do with learning and memory?

One downstream effect of activation of the D1-cAMP-PKA pathway is the activation of CREB. CREB stands for cAMP response element binding protein. CREB can be activated by multiple protein kinases including PKA and several Calcium-dependent protein kinases including calcium/calmodulin-dependent protein kinase type IV (CAMKIV) (Hyman and Malenka, 2001). CREB plays a critical role in learning and memory. For example, overexpression of CREB in the amygdala via viral vector-mediated gene transfer, significantly enhanced long-term memory of associative learning between light and aversive stimulus (shock) (Josselyn et al., 2001). Also, in transgenic mice with CRE-lac Z reporter, auditory cue-fear conditioning enhanced expression of CRE-mediated gene expression in the amygdala (Impey et al., 1998). Further evidence of the role of CREB in learning and memory comes from invertebrate models such as, *Aplysia* and *Drosophila* [for reviews see (Yin and Tully, 1996; Silva and Murphy, 1999)]. Cocaine-associated environments have also been shown to increase levels of phosphorylated CREB (active form) in the nucleus accumbens (Miller and Marshall, 2005a). Based on this and evidence from other associative learning paradigms, CREB may play an important role in cocaine-associative learning. However, cocaine-induced elevation of CREB is believed to reduce the rewarding effects of cocaine (Carlezon et al., 1998).

Another downstream substrate of D1-cAMP-PKA is DARPP-32 (dopamine and cyclic AMP regulated phosphoprotein) (Greengard, 2001). Activation of cAMP dependent PKA, phosphorylates DARPP-32 at Thr34, and this phosphorylated form of DARPP-32 inhibits protein-phosphatase-1 (Hemmings et al., 1984). Protein phosphatase-1 regulates the functioning of a number of voltage-gated and ligand-gated ion channels such as GABA_A, NMDA and AMPA receptors (Greengard et al., 1999). In addition, protein phosphatase-1 is also known to inhibit phosphorylated form of CREB (Bourtchuladze et al., 1994). Thus, inhibition of protein phosphatase-1 via DARPP-32, can play an important role in cocaine and DA mediated plasticity. DARPP-32 knockout mice show reduced cocaine-

induced place preference (Zachariou et al., 2002), which suggests that DARPP-32 may play a role in cocaine-associative learning.

The Fos family of proteins is another important downstream transcription factor of the D1 receptor (Cho et al., 2007). Fos family of proteins form heterodimers with jun family proteins, which bind to activator protein-1 (AP-1) sites present within the promoters of certain genes. The Fos family of proteins is known to play important role in cue-associated cocaine seeking and cocaine conditioned hyperactivity. For example, cues associated with cocaine increase *c-fos* expression in the amygdala during cue elicited cocaine-seeking (Miller and Marshall, 2005b). Also, exposure to cocaine-paired environments increases *c-fos* expression in the cingulate cortex, amygdala, paraventricular nucleus of the thalamus and lateral septal nucleus (Brown et al., 1992). An important member of the Fos family transcription factors, that is believed to play a critical role in long-term adaptation, is delta Fos B (McClung et al., 2004). Levels of delta Fos B are up-regulated following repeated cocaine administration. It is the longest-lasting molecular change in the brain seen following repeated exposure to drugs of abuse, such as cocaine (Nestler, 2001). Delta Fos B, though, has not been shown to be associated with cocaine-associative learning. The above-mentioned molecular substrates are very transient and it is difficult to reconcile these transient changes with more permanent changes in behavior. Thus, the hunt must continue for more long lasting molecular substrates, which may play a critical role in cocaine-associative learning. In addition to these molecular substrates, DA and dopaminergic receptors also play a role in mediating synaptic plasticity.

1-3-4. Role in Synaptic Plasticity (LTP and LTD)

It is widely believed that long-lasting, activity dependent changes at the synaptic level (synaptic plasticity) play an important role in the process of associative learning (Martin

et al., 2000; Maren, 2001), including drug-associative learning (Berke and Hyman, 2000; Hyman and Malenka, 2001; Hyman et al., 2006). The two most common forms of long-term synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD) (Malenka and Bear, 2004). Synaptic plasticity can either strengthen synapses (LTP) or weaken them (LTD). Strengthening of synapses can lead to increase in release of neurotransmitters, formation of new synapses, insertion of new receptors, while weakening of synapses can lead to opposite changes. Till recently, there was no direct evidence to show that synaptic plasticity, occurring at certain synapses in certain brain regions, is essential for associative learning. In 2005, Malinow and colleagues showed that cue-electric shock associative learning can be disrupted by blocking LTP generation in lateral amygdala neurons (Rumpel et al., 2005). In this elegant study, they first tagged GluR1 subunit of the AMPA receptors using viral vectors in the lateral amygdala neurons (a region known to be involved in fear conditioning) of rats. They, then exposed one group of the animals to cue-shock conditioning trials, while control groups were exposed to either shock or cue independently. They observed, that animals that underwent cue-shock associative training showed greater insertion of tagged AMPA receptors as compared to control groups. They also went on to show, that cue-shock associative memory retention in rats is affected by disrupting LTP through viral-mediated insertion of defective subunits of the AMPA receptor.

Both LTP and LTD occur widely at excitatory synapses and DA has a modulatory role in generation of these forms of synaptic plasticity (Jay, 2003). Most of the evidence with respect to the role of DA in LTP and LTD comes from corticostriatal synapses (Calabresi et al., 1997a; Calabresi et al., 2007). For example, induction of both LTP and LTD in corticostriatal slices is blocked by depletion of DA using the pharmacological agent 6OHDA (Calabresi et al., 1992b; Centonze et al., 1999). Both, D1 and D2 receptors contribute to LTD, which can be blocked by both D1 and D2 antagonists (Calabresi et al., 1992b). In addition, in dopamine-depleted slices, it was possible to restore LTD by co-

administration of D1 and D2 agonists (Calabresi et al., 1992a). In the induction of LTP, D1 and D2 receptors may play opposing roles. For example, D2 antagonists enhance induction of LTP (Centonze et al., 2004), while both D1 and D5 antagonists have been shown to block LTP induction (Kerr and Wickens, 2001). In addition, LTP induction is lost in mice lacking D1 receptors (Centonze et al., 2003). Thus, from these studies it is clear, that DA and its receptors play an important role in learning and experience-dependent plasticity at the synaptic level. The conditions under which DA promotes one form of plasticity over another are not known. It is postulated that the specific type of synaptic plasticity is determined by the pattern of DA release and the location of receptors activated (Calabresi et al., 2007).

The nucleus accumbens (NAcc) a focus of this dissertation plays an important role in cocaine reinforcement, cocaine-associative learning and cocaine-induced behavioral sensitization (see section 1-4). The NAcc is also key site for cocaine-induced synaptic plasticity. Both LTP and LTD have been shown to occur in the NAcc following repeated cocaine administration and have been suggested to play an important role in behavioral sensitization seen following repeated cocaine exposure. For example, it has been shown that repeated cocaine administration leads to an upregulation of NAcc AMPA receptor expression (Boudreau and Wolf, 2005) and long-term potentiation at corticoaccumbal synapses (Yao et al., 2004). These studies suggest that this potentiation of glutamatergic transmission in the NAcc (LTP) is critical for behavioral sensitization. In contrast, Thomas et al (2001) have shown that repeated cocaine administration leads to depression in glutamatergic synaptic transmission (LTD) (Thomas et al., 2001) and blockade of this cocaine-induced LTD disrupts cocaine-induced behavioral sensitization (Brebner et al., 2005). At this juncture, it is not clear which form of synaptic plasticity is more critical for long-term behavioral plasticity seen following repeated cocaine administration. It is possible that both these forms of synaptic plasticity do occur and differences in findings could be due to differences in regions sampled. For example, LTD reported by Thomas

et al. (2001) was demonstrated mostly in the shell region of the NAcc. In contrast, Boudreau et al (2005) demonstrated an upregulation of AMPA receptors mainly in the core region of the NAcc. Another reason for the differences could be because of different portions of the cell sampled for example distal dendrites vs. soma of the cell. A recent paper has suggested that cocaine experience may result in bidirectional synaptic plasticity and the type of plasticity may be determined by the history of recent cocaine exposure (Kourrich et al., 2007). For example, in this paper the authors demonstrate that during withdrawal from repeated cocaine exposure there is upregulation of excitatory synaptic currents in the NAcc. However, a single re-exposure to cocaine during extended withdrawal can lead to synaptic depression and abrupt cessation of the initial potentiation. Although, mechanisms of this reversal are not entirely clear, it is postulated that supraphysiological activation of NAcc glutamate transmission may lead to AMPA receptor endocytosis and subsequent synaptic depression. Following this very brief review of synaptic plasticity in associative learning and cocaine-induced behavioral sensitization, brain dopaminergic pathways will be reviewed.

1-3-5. Brain Dopaminergic Pathways

DA was initially thought to be only a precursor of norepinephrine (NE) and epinephrine (E), but its unique distribution as compared to NE suggested that it may have its own unique role. There are several more mesencephalic dopaminergic neurons (15,000 – 20,000) on each side of the midline as compared to noradrenergic neurons (5000 on each side). Based on the length of the efferent DA fibres, dopaminergic systems (Cooper et al., 2003) have been classified into :

1) Ultrashort systems:

- a) Interplexiform amacrine-like neurons: these link the inner and outer plexiform layers of the retina.

- a) Periglomerular DA cells of the olfactory bulb: these link together mitral cell dendrites in separated adjacent glomeruli.

1) Intermediate length system:

- a) Tuberohypophysial DA cells: these project from the arcuate and periventricular nuclei into the intermediate lobe of the pituitary and into the median eminence.
- a) Incertohypothalamic neurons: these link the dorsal and posterior hypothalamus with the dorsal posterior hypothalamus and lateral septal nuclei.
- a) Medullary periventricular group: these include DA cells in the perimeter of the dorsal motor nucleus of the vagus nerve, the nucleus tractus solitarius and the cells scattered in the tegmental radiation of the periaqueductal gray matter.

1) Long systems

The long systems are the long projections originating from the from the ventral tegmental area (VTA) (A8, A10) and substantia nigra (A9) DA cells. These systems can be divided into

- a) Mesolimbic pathway consisting of projections originating from the VTA dopaminergic cells and projecting to the limbic structures (nucleus accumbens, olfactory tubercle, amygdaloid complex and piriform cortex)
- a) Mesocortical pathway consisting of projections from the VTA dopaminergic cells to the limbic cortex (medial prefrontal, cingulate and entorhinal areas).
- a) Nigrostriatal pathway consisting of projections from the substantia nigra dopaminergic cells project to the striatum (caudate and putamen).

Among all these pathways, the mesolimbic pathway, originating from the VTA and terminating in the NAcc, is of most importance in this dissertation and hence will be discussed in greater detail.

1-4. Mesolimbic Dopaminergic System

The mesolimbic dopaminergic system is an important pathway, and is also sometimes called as the reward pathway. It is called so, because it mediates the reinforcing effects of both natural rewards and drugs of abuse. In the following sections, first the origin (VTA) and an important terminal region (NAcc) will be discussed. Following that, evidence with respect to the role of the mesolimbic dopaminergic system in reinforcement and conditioning will be presented. Finally, the role of the mesolimbic dopaminergic system in mediating reward prediction error and uncertainty will be discussed.

1-4-1. Ventral Tegmental Area (VTA)

The VTA lies medial to the substantia nigra and ventral to the red nucleus in the midbrain (Paxinos and Watson, 1998). The total number of neurons in the rat VTA is about 30,000 and unlike the adjacent substantia nigra (90%), fewer than 60% of them are dopaminergic neurons (Swanson, 1982; Margolis et al., 2006a). The non-dopaminergic neurons are primarily GABAergic neurons and they constitute about 15-20% of all the cells in the VTA (Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000). Recent evidence, suggests that some of the VTA neurons also release glutamate (Chuhma et al., 2004; Lavin et al., 2005).

The VTA receives glutamatergic afferents from the prefrontal cortex (PFC), lateral hypothalamus, bed nucleus of stria terminalis and the superior colliculus (Sesack and Pickel, 1992; Georges and Aston-Jones, 2002; Rosin et al., 2003; Geisler and Zahm,

2005). The VTA also receives significant glutamatergic, GABAergic and cholinergic input from the pedunculopontine (PPTg) and laterodorsal tegmental nucleus (LDT) (Cornwall et al., 1990; Semba and Fibiger, 1992; Oakman et al., 1995). The VTA receives predominantly GABAergic afferents from the nucleus accumbens (NAcc) and ventral pallidum; serotonergic afferents from the dorsal raphe nucleus and noradrenergic input from the locus coeruleus (Phillipson, 1979; Overton and Clark, 1997; Geisler and Zahm, 2005). The VTA sends dopaminergic projections to the NAcc, PFC, amygdala, hippocampus, lateral hypothalamus, entorhinal cortex and the lateral septal area (Berger et al., 1974; Fallon and Moore, 1978; Lindvall et al., 1978; Lindvall and Stenevi, 1978; Beckstead et al., 1979). The projections to the NAcc (65%-85%) are richest in DA followed by the projections to the lateral septal area (72%), amygdala (53%), PFC (30%-40 %) and hippocampus (6%-8%) (Swanson, 1982; Gasbarri et al., 1994; Carr and Sesack, 2000; Margolis et al., 2006b). The VTA also sends significant GABAergic efferents to the NAcc and PFC (Margolis et al., 2006b). Electrophysiological studies indicate that the VTA also sends some glutamatergic projections to the NAcc and PFC (Chuhma et al., 2004; Lavin et al., 2005).

Midbrain dopaminergic neurons (VTA) display three main patterns of activity: an inactive hyperpolarized state; a slow (2-10 Hz), irregular, single-spike or 'tonic' firing pattern; a burst or 'phasic' mode (Grace and Bunney, 1983). Single-spike or tonic firing is driven by intrinsic pacemaker potential, while burst firing is dependent on excitatory afferent input (Overton and Clark, 1997). At basal levels, the DA neurons can fire at frequencies of around 5 Hz, but can burst with a transient rate of firing of 30 Hz (Grace, 1987). The spontaneous tonic firing maintains a stable level of DA in the terminal areas and is known as the 'tonic' DA state (Grace, 1991). On the other hand, burst firing is responsible for increased synthesis and release of DA in the terminal areas such as the NAcc and PFC, and plays an important role in goal directed behavior (Floresco et al., 2003). Normally, more than 50% of the dopaminergic neurons are not spontaneously

active (Grace and Bunney, 1984) and are inhibited by GABAergic input from the ventral pallidum (Grace and Bunney, 1985). The hippocampus regulates the activity of midbrain dopaminergic neurons indirectly through the NAcc (Floresco et al., 2001). Activation of the ventral subiculum of the hippocampus activates the NAcc neurons, which leads to inhibition of the ventral pallidum. This in turn makes the midbrain dopaminergic neurons spontaneously active. Glutamatergic inputs to the midbrain dopaminergic neurons is critical for burst firing but can occur only in spontaneously active neurons (Floresco et al., 2003).

1-4-2. Nucleus Accumbens (NAcc)

As previously mentioned, the NAcc is an important part of the mesolimbic pathway and receives dopaminergic neurons from the VTA (Fallon and Moore, 1978). The neurons in the NAcc are mostly GABAergic (90-95%) and are called medium spiny neurons because of their characteristic spiny appearance and size. The other types of neurons in the NAcc are the large aspiny cholinergic interneurons, medium sized aspiny GABAergic and somatostatinergic interneurons (Chang et al., 1982; Chang and Kitai, 1985). Each of these constitutes 1-3% of all striatal neurons. All these neuronal subtypes innervate the medium spiny neurons (Izzo and Bolam, 1988). There is also cross-talk among these interneurons (Koos and Tepper, 1999, 2002). The large cholinergic interneurons receive glutamatergic inputs from the thalamus and dopaminergic inputs from the midbrain. They also influence the release of DA from the dopaminergic axon terminals (Zahm, 1999; Wonnacott et al., 2000). The small GABAergic interneurons provide one source of inhibitory synaptic input to the medium spiny neurons and most likely inhibit other interneurons. The somatostatin-positive interneurons are thought to have a primarily modulatory role (Koos and Tepper, 1999).

Among all the neurons the medium spiny GABAergic neurons receive the greatest extrinsic and intrinsic inputs (hence the importance of its typical morphology) and they also form the main output of the NAcc. The major inputs to the NAcc come from the VTA, PFC, amygdala, thalamus, hippocampus (subiculum), raphe nucleus and brain stem autonomic centers (Beckstead et al., 1979; Kelley and Domesick, 1982; Kelley et al., 1982; McDonald, 1991; Brog et al., 1993). These inputs carry a lot of information from areas involved in processing of memory and sensory information (Kelley, 1999). In return, the NAcc sends projection neurons to the globus pallidus, entopeduncular nucleus, hypothalamus, amygdala and VTA (Nauta et al., 1978; Groenewegen and Russchen, 1984; Heimer et al., 1991). The projection areas are responsible for skeletal and visceral motor responses, and provide feedback information for future modification of sensory and memory information. Thus, the NAcc forms a critical structure integrating sensory and motor information. It also provides feedback information to the sensory and memory areas for more efficient responses in the future. This important role requires special organization of synaptic input on the spinous processes of the medium spiny neurons. The glutamatergic input coming from the cortex and thalamus synapse with medium spiny neurons on the outer two-thirds of the spinous processes. In contrast, the dopaminergic input coming from the VTA synapse with the medium spiny neurons at the necks of the spinous processes (Sesack and Pickel, 1990; Smith and Bolam, 1990).

The NAcc can be divided into core and shell regions on the basis of distribution pattern of various neuroactive substances (Zaborszky et al., 1985; Voorn et al., 1989).

Histological staining methods such as acetylcholinesterase, choline acetyltransferase and neurotensin immunohistochemical methods produce denser patterns in the shell as compared to the core (Zahm and Brog, 1992; Brog et al., 1993; Heimer et al., 1993). In addition, the shell has higher concentration of 5-HT₄ serotonergic receptors (Patel et al., 1995), D₃ receptors (Le Moine and Bloch, 1996) and cocaine-and amphetamine-regulated transcript (CART) transcript and its peptide (Koylu et al., 1998).

Cytoarchitecturally, the shell is described as a cell-poor region with a loose arrangement of neurons, while the core has a more densely packed arrangement of neurons (Herkenham et al., 1984). The core and shell also have different afferent and efferent connections (Heimer et al., 1991; Zahm and Brog, 1992; Brog et al., 1993). For example, the prefrontal cortex projections to the shell come from the infralimbic and posterior piriform cortices, while projections to the core come from dorsal prelimbic, anterior agranular insular, anterior cingulate and perirhinal cortices. Similarly, though both core and shell receive input from the hippocampus, the ventral subiculum projects exclusively to the shell, while the dorsal subiculum projects to the core. Also, the input from the amygdala to the shell comes primarily from the central nucleus of the amygdala, while the core receives input from the basolateral nucleus of the amygdala. In terms of efferent connections, the core projects more to the classic basal ganglia output structures such as the substantia nigra, ventral pallidum and subthalamic nucleus. On the other hand, efferents from the shell project preferentially to the subcortical limbic regions, such as the lateral hypothalamus, ventral tegmental area, ventromedial ventral pallidum and brain stem autonomic centers. The functional roles of the core and shell are not very clear and new anatomical evidence suggests that the shell and core may be communicating with each other (Haber et al., 2000). It is possible, that through these communications the core and the shell are able to influence each other's output. In this dissertation, the core and shell were not differentiated and microdialysis probes included both regions.

1-4-3. Role in Reinforcement

Several studies have shown that the mesolimbic DA system is activated following natural rewards. For example, single unit recordings in non-human primates have shown that VTA dopaminergic neurons show phasic activation following receipt of food reward (Ljungberg et al., 1992; Schultz et al., 1993; Mirenowicz and Schultz, 1994; Schultz, 2001). An increase in NAcc DA following a food reward has also been demonstrated

using *in-vivo* microdialysis (Hernandez and Hoebel, 1988a; Radhakishun et al., 1988; Westerink, 1995).

In addition to appetitive rewards, the mesolimbic dopaminergic system is involved in mediating the reinforcing effects of drugs of abuse such as cocaine. For example, cocaine self-administration, which models the reinforcing effects of cocaine, is disrupted following lesions of NAcc dopaminergic terminals (Pettit et al. 1984; Zito et al., 1985; Caine & Koob, 1994) or VTA dopaminergic cell bodies (Roberts & Koob, 1982). Similarly, *in-vivo* microdialysis (Hurd et al., 1989; Ikegami and Duvauchelle, 2004b) and voltammetry (Kiyatkin et al., 1993) studies have demonstrated an enhancement in NAcc DA following cocaine self-administration. In addition, it has also been reported that animals resume lever response during cocaine-self-administration sessions, when NAcc DA levels fall below a threshold level (Kiyatkin and Stein, 1995; Wise et al., 1995; Ranaldi and Roberts, 1996).

However, the mesolimbic dopaminergic system is activated not only by positive reinforcement, but also responds to aversive stimuli. For example, midbrain dopaminergic neurons are activated by aversive stimuli (Kiyatkin and Zhukov, 1988). In addition, increase in NAcc DA has been reported using aversive stimuli such, as mild foot shock (Sorg and Kalivas, 1991; Young et al., 1993) and tail pinch (Louilot et al., 1986). Restraint stress also shows an increase in NAcc DA (Imperato et al., 1991; Doherty and Gratton, 1997; Weiss et al., 1997) also see review (Joseph et al., 2003). Thus, it is clear that the mesolimbic dopaminergic system is activated by both positive-reinforcement and aversive stimuli.

1-4-4. Responses to Conditioned Stimuli

The mesolimbic dopaminergic system responds to stimuli consistently associated with food. For example, when stimuli are consistently associated with reward, midbrain dopaminergic neurons are activated in response to stimuli signaling the occurrence of future reward (Ljungberg et al., 1992). Similarly, increase in NAcc DA has also been demonstrated in response to cues signaling delivery of food reward, using techniques such as voltammetry (Phillips et al., 1993; Kiyatkin and Gratton, 1994; Richardson and Gratton, 1996) and *in-vivo* microdialysis (Datla et al., 2002).

Stimuli consistently associated with drug reward also enhance NAcc DA. For example, using place-conditioning and *in-vivo* microdialysis, significantly greater enhancement in NAcc DA is seen in cocaine-associated contexts as compared to saline-associated contexts (Duvauchelle et al., 2000b; Bassareo et al., 2007). In addition, conditioning studies using cues prior to cocaine self-administration have reported an increase in NAcc DA following presentation of cues associated with cocaine, but before making operant responses for cocaine (Gratton and Wise, 1994; Kiyatkin and Stein, 1995; Ito et al., 2000; Phillips et al., 2003). Studies have also reported activations of NAcc neurons, following presentation of cues associated with cocaine (Carelli and Ijames, 2001; Hollander and Carelli, 2007). Thus, with repeated association of stimuli with reinforcers, stimuli gain salience. These salient stimuli activate the mesolimbic dopaminergic system and signal the occurrence of a forthcoming reinforcer such as reward or punishment.

1-4-5. Role in Pavlovian and Instrumental Conditioning

The mesolimbic dopaminergic pathway also plays a critical role during conditioning (Pavlovian and instrumental). For example, Young and his associates showed that NAcc DA is increased during each pairing of contextual stimuli with the aversive stimuli

(Young et al., 1998). In Pavlovian conditioning paradigms, acquisition of conditioned approach towards stimuli signaling delivery of food is blocked by 6 OHDA lesions of the dopaminergic nerve terminals in the NAcc (Dalley et al., 2002; Parkinson et al., 2002). Similarly, approach responses in Pavlovian conditioning paradigms are also blocked by systemic D1/D2 antagonist (alpha-flupenthixol) pretreatment prior to conditioning trials (Di Ciano and Everitt, 2001). Systemic administration of D1 antagonists have also been shown to disrupt appetitive learning (Beninger and Miller, 1998; Azzara et al., 2001; Eyny and Horvitz, 2003). Interestingly, systemic D2 antagonists have been shown to enhance appetitive learning (Eyny and Horvitz, 2003). However, this is not in conflict with the role of DA in conditioning, as D2 antagonists can enhance the release of DA by acting on dopaminergic autoreceptors. Also, D2 antagonists have opposite effects on DARPP-32 and striatal plasticity (LTP) as compared to D1 antagonists (Calabresi et al., 1997b). Dopaminergic antagonists also block the acquisition of lever responses in instrumental conditioning paradigms involving food and water reward (Wise and Schwartz, 1981). In animals that have already acquired lever responding, dopaminergic antagonists cause a decrease in responding, and the animal behaves as if it is undergoing extinction even though it is receiving the reward (Wise, 2004).

The role of DA in mediating cocaine-induced CPP is not very clear. For example, although pretreatment with DA release inhibiting agent (CGS 10746B) has been shown to block the acquisition of cocaine-induced CPP (Bilsky et al., 1998), 6-OHDA lesions of dopaminergic nerve terminals did not block cocaine-induced CPP (Spyraki et al., 1982). Also, although the D1 antagonist SCH 23390 (Cervo and Samanin, 1996) has been shown to block cocaine-associative learning, most D2 receptor antagonists such as haloperidol (Mackey and van der Kooy, 1985), alpha-flupenthixol (Mackey and van der Kooy, 1985), pimozide and sulpiride failed to block the acquisition of cocaine-induced CPP. However, intravenous cocaine-induced CPP was blocked by DA receptor antagonist haloperidol (Spyraki et al., 1987).

1-4-6. Role in Conditioned Reinforcement, Pavlovian Instrumental Transfer and Latent Inhibition Paradigms

The role of DA in the process of conditioning has also been shown using a number of conditioning paradigms, such as conditioned reinforcement, Pavlovian instrumental transfer and latent inhibition paradigms. In the conditioned reinforcement paradigm, the animal is trained to associate a stimulus such as light or tone with a primary reward such as food/water (Robbins and Everitt, 1982; Ikemoto and Panksepp, 1999). Subsequently, the ability of the conditioned stimulus to act as a reinforcer in the acquisition of an instrumental response is examined. The animals thus learn a new response, reinforced solely by a secondary reward and not the primary reward. Increase in intracumbal DA using amphetamine microinjections can increase conditioned reinforcement (Taylor and Robbins, 1984; Kelley and Delfs, 1991). Infusion of D1 and D2 antagonists into the NAcc block amphetamine induced enhancement of conditioned reinforcement (Wolterink et al., 1993). Similarly, amphetamine induced enhancement in conditioned reinforcement is also blocked by 6-OHDA lesions in the NAcc (Taylor and Robbins, 1986).

Pavlovian instrumental transfer is another paradigm used to assess associative learning (Lovibond, 1983; Balleine, 1994; Rescorla, 1994). This paradigm has two distinct phases: the first phase, the animals are trained associate a stimulus (X) with food reward (Pavlovian conditioning). In the second phase, the animals are trained to obtain the same food reward by performing a task such as lever press in the absence of the stimulus (X) (instrumental conditioning). Finally, on the test day the animal is allowed to lever press under extinction conditions i.e. no food reward is delivered upon lever response. During the test session the conditioned stimulus (X) is presented intermittently and the presentation of conditioned stimulus (X) potentiates the lever responding by the animal. This test assesses the ability of the animal to associate the stimulus (X) with the instrumental behavior, which has been rewarded with food during instrumental

conditioning. Several studies have found that NAcc DA plays a critical role in this type of associative learning. For example, amphetamine-induced increases in NAcc DA enhances the CS-induced potentiation (Wyvell and Berridge, 2000). Also, cytotoxic lesions of NAcc abolished Pavlovian instrumental transfer (Corbit et al., 2001; de Borchgrave et al., 2002).

Latent inhibition is defined as the attenuated ability to form associative relationship between a familiar stimulus and an unconditioned reinforcer. In other words, pre-exposing the stimulus in the absence of reinforcer makes it difficult for the animal to associate the stimulus with the reinforcer (Gray et al., 1995; Gray et al., 1997; Weiner and Feldon, 1997). Increasing NAcc DA by intracumbal amphetamine injections disrupts latent inhibition (Solomon and Staton, 1982). In other words, increased DA in the NAcc helps the animal to associate previously exposed stimulus with reward. Also, destruction of NAcc DA terminal using 6-OHDA or injections of the D2 antagonist haloperidol enhance latent inhibition (Gray et al., 1997; Ruob et al., 1997). All the above evidence suggests, that mesolimbic DA plays a critical role in the process of associative learning. However, according to contemporary learning theories, uncertainty and prediction errors play an important role in associative learning. Does the mesolimbic dopaminergic pathway mediate reward-prediction error and uncertainty?

1-4-7. Role in Reward Prediction Errors and Uncertainty

Midbrain dopaminergic neurons respond to reward-prediction errors (Montague et al., 1996; Schultz et al., 1997; Schultz and Dickinson, 2000). Studies using single unit recordings in non-human primates showed that initially dopaminergic neurons respond to the unpredicted occurrence of rewards (Mirenowicz and Schultz, 1994; Schultz, 1994). But as stimuli are presented consistently prior to the occurrence of rewards, midbrain dopaminergic neurons start responding to the stimuli in expectation of rewards, rather

than to the rewards themselves (Ljungberg et al., 1992; Schultz et al., 1993). Firing of midbrain dopaminergic neurons is depressed, when expected rewards fail to occur (negative prediction error). Similarly, if rewards occur when no reward is expected (positive prediction error), midbrain dopaminergic neurons showed phasic increase in firing (Hollerman and Schultz, 1998; Satoh et al., 2003). Thus, dopaminergic neurons respond to both, positive and negative prediction errors. In humans, using functional neuroimaging (fMRI), the NAcc has been shown to play an important role in mediating reward prediction errors. For example, fMRI studies using monetary reward have shown that the NAcc is activated by occurrence of unexpected reward, and depressed when expected rewards failed to occur (Abler et al., 2006; Spicer et al., 2007).

Another study, conducted by Fiorillo et al. showed that midbrain dopaminergic neurons respond to reward uncertainty (Fiorillo et al., 2003). In this study, they associated five different visual stimuli with reward (fruit juice) to a different extent. Each stimulus predicted the occurrence of the reward either 0, 25%, 50 %, 75% or 100% of the time. Stimuli that predicted the occurrence of reward 25%, 50% and 75% of the time induce uncertainty with respect to the occurrence of reward, and midbrain dopaminergic neurons show sustained firing from the point of introduction of these cues till the occurrence of reward. More importantly, this firing of dopaminergic neurons is maximal in the presence of stimuli equally (50%) associated with reward and non-reward. Theoretically, maximum uncertainty occurs when there is equal chance of receiving reward and non-reward. This study, showed that maximal uncertainty results in maximal activation of midbrain dopaminergic neurons. Recent fMRI studies in human volunteers also suggest that the mesolimbic dopaminergic pathway is involved in mediating uncertainty associated with reward outcome (Berns et al., 2001; Abler et al., 2006; Dreher et al., 2006). Experimental evidence from animal and human studies clearly implicates the mesolimbic dopaminergic pathway as a mediator of uncertainty and prediction errors

involving natural rewards. Thus the following question arises, can these findings from natural rewards be extrapolated to drug rewards such as cocaine?

1-5. Drug Rewards vs. Natural Rewards: Similarities & Differences

Both, drugs of abuse (cocaine, MDMA etc.) and natural rewards (food, water, sex, money) are classified as positive reinforcers i.e. they both cause approach behavior, they both cause animals to repeat their actions and maintain behavior and finally, both forms of reinforcers lead to feelings of pleasure and happiness (Di Chiara et al., 1999). Both, drug and natural rewards cause increase in NAcc DA (Hernandez and Hoebel, 1988b). However, the manner in which these rewards increase DA is different. The increase in DA is much less with natural rewards as compared to drug rewards. Also, the increase in DA is more sustained with drugs of abuse as compared to natural rewards (Di Chiara et al., 1999). The DA response following receipt of natural reward tends to habituate over time, which is not the case for drug reward. Another characteristic feature of drug reward different from natural reward is the resistance to extinction i.e. behavior initiated to obtain the reward (for e.g. lever response during self-administration) can be maintained for a prolonged period of time, even though the action does not actually lead to reward (Miles et al., 2003; Ciccocioppo et al., 2004). In case of natural reward, if reward is not obtained the action undergoes extinction faster than for drug rewards. One more feature, characteristically seen with drug reward, is the phenomenon of sensitization.

Sensitization is the enhancement in response seen following repeated exposure to the drug of abuse, even though the dose of the drug remains the same (Kalivas and Duffy, 1993; Vanderschuren and Kalivas, 2000). In contrast, natural rewards often show tolerance or habituation. An important difference between natural and drug rewards is that the chance of getting dependent is much higher with drug reward than with natural reward. A few reports do suggest the development of dependence on food reward (Wang et al., 2004; Carlezon and Chartoff, 2007; Teegarden and Bale, 2007). Chronic exposure

to drugs leads to morphological changes in neurons in areas rich in DA, something that has not been shown with natural rewards. For example, neuronal dendritic branching and spine density in the NAcc and PFC is enhanced following exposure to drugs of abuse as compared to controls exposed to natural rewards (Robinson et al., 2001). These changes in neuronal structure are likely to be responsible for some of the behavioral and neural plasticity changes seen in animals exposed to drugs of abuse. Keeping the similarities and all the differences mentioned above in mind, it is essential to assess the influence of uncertainty and prediction errors on cocaine-associative learning.

1-6. Cocaine-Associative Learning: Uncertainty and Prediction Error?

To induce states of ‘Uncertainty’ and ‘Prediction Error’ with respect to cocaine reward, it was first essential to condition the animals using cue-associative training sessions. Expectations of cocaine reward were induced by consistently associating distinct cues (visual + olfactory) with cocaine and saline self-administration sessions. This cue-associative training was called ‘Certainty’ cue-associative training. In contrast, cues were associated equally with cocaine and saline to induce ‘Uncertainty’ with respect to cocaine reward occurrence. This cue-associative training was called ‘Uncertainty’ cue-associative training (see details in Materials and Methods). Since the influence of ‘Uncertainty’ cue-associative training on behavior has not been reported in literature, our first specific aim was to measure the effects of cue-induced Certainty and Uncertainty on behavioral parameters during cue-associative training sessions (Specific Aim 1). The behavioral parameters used were lever responses for cocaine and saline, and locomotor activity during cocaine and saline self-administration.

Following cue-associative training, the influence of cue-induced Certainty, Uncertainty and Prediction Error on NAcc DA and locomotor activity following self-administration of cocaine and saline (Specific Aim 2). The NAcc was chosen as a target, because it is an

important terminal region of VTA dopaminergic neurons (Fallon and Moore, 1978). Also, the NAcc plays an important role in mediating cocaine reinforcement (Koob, 1992b). In addition, studies have shown that NAcc DA is enhanced in the presence of cues consistently associated with cocaine (Kiyatkin and Stein, 1996; Di Ciano et al., 1998). Most importantly, fMRI studies in human volunteers have shown that NAcc is involved in ‘Prediction Error’ and ‘Uncertainty’ associated with monetary reward (Matthews et al., 2004; Dreher et al., 2006).

NAcc DA was measured using *in-vivo* microdialysis with sample collection every 10 minutes. ‘Certainty’ with respect to cocaine was induced by allowing animals to self-administer cocaine in the presence of cues consistently associated with cocaine. On the other hand, ‘Prediction Error’ was induced by allowing animals self-administer cocaine in the presence of cues consistently associated with saline. ‘Uncertainty’ was induced in animals from the ‘Uncertainty’ cue-associative training group, using cues equally associated with cocaine and saline. Similarly ‘Certainty’, ‘Prediction Error’ and ‘Uncertainty’ with respect to non-reward was induced by allowing animals to self-administer saline in the presence of cues consistently associated with saline, consistently associated with cocaine and equally associated with cocaine and saline respectively (Details in Materials & Methods section).

1-7. Cocaine and Locomotor Activity

Cocaine and other psychostimulants enhance locomotor activity in drug naïve animals. Following repeated administration of cocaine, there is usually an enhancement in locomotor activity depending on administration schedule and cocaine dose used (Kalivas and Duffy, 1993). This enhancement in locomotor activity following repeated exposures is termed as locomotor sensitization. Finally, studies have also shown that locomotor activity in rodents is increased in the presence of cues associated with cocaine. This

increase in locomotor activity is called conditioned increase in locomotor activity. Both, locomotor sensitization and conditioned increase in locomotor activity are learned phenomenon, and are influenced by the environment in which cocaine is administered. The following sections will discuss the role of the mesolimbic dopaminergic system in mediating the three types of locomotor activity.

1-7-1. Cocaine-Induced Increase in Locomotor Activity

The mesolimbic dopaminergic system plays an important role in mediating the increase in locomotor activity following administration of psychostimulants like cocaine. For example, cocaine administration results in increased locomotor activity and NAcc DA levels (Hurd et al., 1989; Ikegami and Duvauchelle, 2004b). Furthermore, pharmacological destruction of mesolimbic dopaminergic terminals abolishes cocaine-induced increase in locomotor activity (Kelly and Iversen, 1976). Micro-injections of cocaine in the NAcc also enhances locomotor activity, and this increase can be blocked by systemic injections of non-selective dopaminergic antagonist cis-flupenthixol (Delfs et al., 1990). Finally, increase in locomotor activity following systemic cocaine can be blocked by intra-accumbens injections of the D2 selective antagonists eticlopride and sulpiride (Neisewander et al., 1995) and the D1 antagonist SCH 23390 (McGregor and Roberts, 1993). Other regions, such as the dorsal striatum, VTA and olfactory tubercle, are also believed to play an important role in mediating the locomotor effects of cocaine (Mayfield et al., 1992; Gong et al., 1996; Borgland et al., 2004; Chambers et al., 2004).

1-7-2. Locomotor Sensitization

Locomotor sensitization is the progressive and enduring augmentation in locomotor activity seen on repeated administration of psychostimulants like cocaine (Kalivas and

Duffy, 1993; Stewart and Badiani, 1993; Vanderschuren and Kalivas, 2000). Both, VTA and NAcc, play an important role in development of behavioral sensitization. For example, studies have shown that locomotor sensitization can be induced by intra-VTA injections of cocaine and dopamine uptake inhibitor GBR 12909 (Cornish and Kalivas, 2001). Similarly, intra-accumbens injections of cocaine and D1 agonist SKF 81298 result in the development of sensitization to cocaine (Hooks et al., 1993; De Vries et al., 1998). Studies have also shown that the development of locomotor sensitization to cocaine can be blocked by intra-VTA injections of NMDA receptor antagonists CPP and MK-801 (Kalivas and Alesdatter, 1993). Behavioral sensitization following systemic administration of cocaine, also results in enhanced firing of dopaminergic neurons (Vanderschuren and Kalivas, 2000) and enhanced release of NAcc DA (Akimoto et al., 1989; Kalivas and Duffy, 1990; Pettit et al., 1990a). Several studies have shown that context or environment plays an important role in cocaine-induced behavioral sensitization, which suggests the role of associative-learning in locomotor sensitization (Weiss et al., 1989; Hooks et al., 1993; Badiani et al., 1995; Carey and Gui, 1998; Duvauchelle et al., 2000b). Repeated intraperitoneal cocaine administration has been shown to result in higher brain cocaine levels (Pettit et al., 1990b). So, increased brain cocaine levels is suggested as a possible explanation for behavioral sensitization (Reith et al., 1987). However, increased brain cocaine levels cannot completely explain behavioral sensitization, as behavioral sensitization induced via intravenous cocaine administration dose not result in increased cocaine brain levels (Orona et al., 1994).

1-7-3. Conditioned Increase in Locomotor Activity

An important expression of cocaine-associative learning, is the increase in locomotor activity in the presence of contexts consistently associated with cocaine (Brown and Fibiger, 1992; Burechailo and Martin-Iverson, 1996; Bell et al., 1997; Duvauchelle et al., 2000a). This conditioned increase in locomotor activity is not limited to entire contexts,

and has also been shown in response to discrete stimuli associated with cocaine (Panlilio and Schindler, 1997). The neural substrates involved in conditioned increase in locomotor activity are not entirely known. A couple of studies have successfully shown, that NAcc DA is enhanced during conditioned increase in locomotor activity (Di Ciano et al., 1998; Duvauchelle et al., 2000a). Conditioned increases in locomotor activity is not blocked by dopaminergic antagonists such as haloperidol and pimozide (Beninger and Herz, 1986; Weiss et al., 1989; Martin-Iverson and Reimer, 1994). Other neural substrates, such as glutamate, serotonin, and corticotrophin-releasing factor have also been suggested to play a role in cocaine-induced conditioned increase in locomotor activity (Carey and Damianopoulos, 1994; Damianopoulos and Carey, 1995; DeVries et al., 1998).

1-8. Diazepam

Diazepam belongs to the benzodiazepine group of drugs. Diazepam is a positive modulator of GABA_A receptors, and binds to its benzodiazepine-binding site. Diazepam is highly lipid soluble, and easily crosses the blood brain barrier. Diazepam is known to have sedative, anxiolytic, anticonvulsant, muscle relaxant and memory impairing effects (amnesia). Diazepam induced amnesia generally occurs after administration of the drug and is known as anterograde amnesia (Lister, 1985; Curran, 1991). Amnesia following diazepam pretreatment has been reported both, clinically (Shane, 1971; Hennessy et al., 1991) and in experimental studies (Roy-Byrne et al., 1987; Rich et al., 2006).

1-8-1. Diazepam and Associative Learning

Diazepam pretreatment in animals has been shown to block acquisition of place learning using the Morris water maze. In its simplest form, the task consists of finding a submerged platform in an opaque pool of water following several acquisition trials

(Morris, 1984). Finding the platform is critical for the animal to avoid drowning in the pool of water. During acquisition trials, the platform is placed below the water and acquisition is assessed, by measuring either the distance traveled to reach the platform or the time taken to reach the platform or both. An acquisition criteria is set for both time taken (latency) and distance traveled. Impairments in acquisition of spatial learning, either due to neurological damage or drug treatment, results in animals taking a longer time to reach the platform or travel a greater distance as compared to control animals. In some studies, the time spent by the rat in the quadrant (where the platform was placed during training) on the test day is also taken as measure of place learning. Diazepam pretreatment during acquisition trials in the Morris water maze enhances both, time taken to find the platform and the distance traveled to reach the platform (Arolfo and Brioni, 1991; McNamara and Skelton, 1991; Brioni and Arolfo, 1992). This blockade of acquisition of place learning by diazepam has been reported in the dose range of 1.0 mg/kg to 3.0 mg/kg given intraperitoneally (IP). In one study, it was also reported that animals treated with diazepam in the dose of 0.3 mg/kg do not show bias to quadrant, suggesting that the cognitive effects of diazepam can be seen at this low dose (Arolfo and Brioni, 1991).

Other paradigms, used to assess impairments in the acquisition of associative learning involve using noxious stimuli, such as shock or loud auditory noise. Experiments using shock generally involve avoidance behavior and assess the ability of an animal to avoid a certain behavior formerly paired with shock. Based on whether an animal has to actively do something to avoid shock or control innate behavior to avoid shock, the shock-avoidance paradigms can be classified as active or passive shock avoidance tasks respectively. Diazepam blocks acquisition of both active (Pereira et al., 1988; Pereira et al., 1989; Celik et al., 1999) and passive (Jensen et al., 1979; Decker et al., 1990) shock avoidance behaviors. Another paradigm to assess associative learning in human subjects is the acquisition of muscular startle responses following conditioning with loud auditory

stimuli (Davis et al., 1993). Diazepam pretreatment during conditioning blocks the acquisition of these muscular responses (Scaife et al., 2005). Thus, diazepam has been shown to block acquisition in a number associative learning paradigms.

1-8-2. Diazepam and Cocaine

Diazepam pretreatment (5.0 mg/kg IP) has also been shown to block the development of cocaine-induced place preference (Meririnne et al., 1999). In a recent study, it was reported that diazepam blocks LTP in an *ex-vivo* preparation of VTA dopaminergic neurons taken from animals treated with cocaine for 5-7 days (Liu et al., 2005). One study has shown, that disruption of LTP can block conditioned responses to electric shock (Rumpel et al., 2005). Thus, by disrupting cocaine-induced LTP, it is possible that diazepam may block associative learning between cues and cocaine. It was hypothesized, that if diazepam pretreatment disrupted cue-reward/non-reward associative training then it would also disrupt the influence of cue-induced uncertainty and prediction error on neurochemical and behavioral responses to self-administered cocaine and saline. Therefore for Specific Aim 3, animals were pretreated with diazepam or saline (control) during cue-associative training. Following the cue-associative training, the influence of cue-induced certainty, uncertainty and prediction error on NAcc DA response and locomotor activity to cocaine and saline self-administration was investigated in diazepam and saline pretreated rats. The same paradigm as used in Specific Aim 1 was used for this part of the study. The only difference was that rats were pretreated with either diazepam or saline (control) during cue-associative training. Since, self-administration of cocaine and saline is involved in this paradigm, it was important to select a dose of diazepam that did not have a motor-impairing effect. A previous study done in our lab showed that diazepam in the dose of 0.25 mg/kg reduced response latency for cocaine and enhanced cocaine self-administration (Maier et al., under revision). The enhanced self-administration of cocaine is believed to be due to the anxiolytic effects of diazepam.

Since, 0.25 mg/kg of diazepam did not affect the ability of animals to self-administer cocaine and also had some cognitive effect (anxiolytic), it was decided to use this dose of diazepam. Another study has also shown, that diazepam in the dose of 0.3 mg/kg has some cognitive effect (Arolfo and Brioni, 1991). Importantly, diazepam pretreatment was carried out only during cue-associative training, and not on the dialysis day when NAcc DA was measured.

1.9. Specific Aims

This dissertation had these three specific aims:

Specific Aim 1: To measure the effects of cue-induced ‘Certainty’ and ‘Uncertainty’ on behavioral parameters during cue-associative training.

Specific Aim 2: To measure after cue-associative training, the influence of cue-induced ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ on NAcc DA and locomotor activity following cocaine and saline self-administration.

Specific Aim 3: To measure the influence of diazepam pretreatment during cue-associative training, on NAcc DA response and locomotor activity to cocaine and saline self-administration under conditions of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’.

Chapter 2: Materials and Methods

2-1. General Materials & Methods

Subjects

Male Sprague Dawley rats (Charles River, Houston) weighing approximately 300 g at the beginning of the experiment were used. The rats were housed individually after surgery in polypropylene cages and maintained on a 12 hour reversed light/dark cycle (lights on 7:00 p.m. to 7:00 a.m.). Animals were handled daily for 2 weeks prior to the start of the experiment. Food and water was available ad libitum in the home cage except during the food-training phase.

Apparatus

Food training, self-administration sessions and in vivo microdialysis test sessions were conducted in identical one-lever operant chambers (28 x 22 x 21 cm) located within sound-attenuating compartments. In all chambers, the ceiling, front and back walls were constructed of Plexiglas. The two sidewalls were constructed of metal with a single retractable operant lever on one of the walls. A stimulus light was located above the retractable lever and a house light was located on the opposite metal wall. Three sets of photocells were located on the front and back walls of the chamber, one in the center and the two others at 5 cm from each end. Photobeam breakages within the operant chamber were compiled as locomotor activity units. The injector system was connected to a swivel mounted on a counterbalanced arm at the top of each chamber. One end of the swivel was connected, via polyethylene tubing (Tygon Microbore 1.5 mm o.d.), to a 10 ml syringe mounted on a syringe pump (Razel, Model A, 33.3 rpm). The other side of the swivel was connected via a spring-covered tubing (Plastics One) to the catheter termination mounted on the top of the animal's head. The experimental programs were controlled, and operant

and locomotor data were collected using a Med Pentium 4.3 GHZ computer and Med-PC software.

Drugs

Cocaine hydrochloride (National Institutes of Drug Abuse) was dissolved in 0.9% sterile saline (Abbott Laboratories ,USA). Following drugs were used during surgery– pentobarbital (Abbott Laboratories, IL), chloral hydrate (Spectrum Quality Products, NJ) and atropine (American pharmaceutical partners Inc , IL). Heparin (American Pharmaceutical partners Inc, CA) and Timentin (Glaxo SmithKline, UK) were used after surgery to flush the catheters.

Food Training

Animals were food restricted (≈ 6 g of standard rat chow per day, adjusted as needed to maintain, not decrease body weight) and trained to lever press for food on a FR1 schedule of reinforcement. Each lever response resulted in dispensing one sucrose pellet (45 mg; P.J. Noyes, Lancaster, NH). After the lever press response for food was acquired (approx 3 days), 10-min food-reinforced operant sessions (FR1) were conducted for the next 6 days without food restriction.

Surgery

After the completion of food training sessions, animals were implanted with a chronic silastic intravenous (i.v.) jugular catheter (0.6 mm o.d.) under pentobarbital sodium (Nembutal, 50 mg/kg, i.p.) anesthesia. Atropine sulfate (250 μ g, s.c.) was given prophylactically to prevent respiratory tract secretions. Supplemental chloral hydrate (80 mg/kg, i.p.) was given if necessary, to prolong anesthesia. Catheters were implanted such that the free end of the catheter with a cannula termination (Plastics One) passed subcutaneously on the side of the neck, out an incision in the animal's head and mounted on the skull. Animals were also stereotaxically implanted with a unilateral guide cannula

(21 g) aimed above the NAcc (flat skull; AP: + 1.7 mm; ML: \pm 1.7 mm; DV:- 2.5 mm). The catheter cannula and the guide cannula were affixed to the skull with four stainless steel screws and dental acrylic cement. Animals underwent a minimum of one-week recovery prior to the beginning of the experiments. After the surgery, animals received 0.1 ml of saline containing 67.0 mg/ml of the antibiotic, timentin and 30 U/ml heparin through their i.v. catheters daily for the next week. Animals continued receiving the same solution daily without the timentin component through the duration of the experiment to maintain catheter patency.

2-2. Methods for Specific Aim1

Cue-Associative Training

Animals were weighed daily and cocaine concentrations were altered accordingly for cocaine sessions, so that each lever press resulted in the delivery of 0.5 mg/kg/infusion cocaine hydrochloride in a volume of 0.1 ml of isotonic saline. During saline sessions, an equal volume of saline was infused. After each infusion, there was a 20-sec “time-out” period, during which time the lever was retracted, the stimulus light turned off and no infusions could be delivered. Training consisted of 16 alternating days of cocaine and saline availability (8 sessions each for cocaine and saline) during one-hour conditioning/self-administration sessions. During the first 30 min of each session the chamber was darkened and the lever was retracted, while animals habituated to the neutral environment. After 30 min, the house light was illuminated, sensory cues (see below) and the lever were presented, and cocaine or saline was available upon lever press for the remaining 30 min.

Sensory Cues.

Visual and olfactory environmental cues were introduced into the operant chamber immediately following the 30 min darkened habituation period. Visual cues consisted of

either black or white felt “walls” attached to the sides of the clear plexiglass operant chamber. Olfactory cues consisted of an oil-based scent (e.g., cinnamon or rose) saturated on a cotton ball located under the grid floor of the operant chamber.

Training Assignments

Animals were assigned to undergo ‘Certainty’ or ‘Uncertainty’ cue-associative training. During ‘Certainty’ cue-associative training distinct cue sets (olfactory + visual) were associated with cocaine and saline self-administration sessions. For example, for ‘Certainty’ training, if rose + black cue set (olfactory + visual) was consistently associated with cocaine, then cinnamon + white (distinct set of cues) was consistently associated with non-reward (saline). During ‘Uncertainty’ cue-associative training, each of the two distinct cue sets was equally associated with cocaine and saline sessions. There were two means of ‘Uncertainty’ training. For ‘Uncertainty 1’, the two sets of cues were alternated daily between reinforcement conditions. For example, the cue for cocaine session on Day 1 was used for saline session on Day 4; the cue for saline session on Day 2 was used for cocaine session on Day 3, and so on. The ‘Uncertainty 2’ group had one set of cues (e.g. rose + black) associated with the first four cocaine sessions and another distinct set of cues (e.g. cinnamon + white) associated with the first four saline sessions. For the last eight sessions, these cue sets were switched i.e. rose + black was used for saline sessions and cinnamon + white was used for cocaine sessions.

Statistical Analyses

All data was analyzed using GB stat version 6.5 (Macintosh version). Lever responses’ across training days for the ‘Certainty’ and ‘Uncertainty’ cue-associative training groups was compared using three-way repeated measures ANOVAs (Training X Treatment X Session Days). Training was ‘Certainty’ or ‘Uncertainty’ and Treatment was cocaine or saline. Similarly, locomotor activity in the same training groups and for the same treatments was also analyzed using three-way repeated measures

ANOVAs). Post hoc analyses (Fisher's LSD) were used to detect group or time differences (e.g., at least $p < 0.05$) when main and/or interaction effects were indicated by overall analyses.

2-3. Methods for Specific Aim 2

In Vitro Recovery Calibration

Microdialysis probes were constructed as previously described (Duvauchelle et al., 2000b; Duvauchelle et al., 2000a), with an active membrane length of 2.5 mm at the probe tip. Prior to probe recovery, all probes were flushed with nanopure water. On the day of probe calibration, 1.0 ml gastight Hamilton 1000 series syringes were filled with freshly prepared filtered Ringer's solution (128.3 mM NaCl, 1.35 mM CaCl₂, 2.68 mM KCl, and 2.0 mM MgCl₂; pH=7.3), and pumped through the probe at 1.63 μ l/min, with the probe tips in a beaker containing the Ringer's solution, ascorbate (0.001%), and 5 nM DA, and maintained at 37° C. Perchloric acid solution (1M Na Bisulfite and 0.2 M EDTA in 0.05 N HClO₄) was added into the collecting tubes to prevent DA degradation. Ten-min samples from each probe were collected and assayed by high performance liquid chromatography with electrochemical detection (HPLC-EC). Recovery values for each probe were calculated by comparing the peak heights of samples to those from a standard 1.25 nM DA. The mean \pm SEM recovery of probes used in the experiment was 14.04 \pm 0.6%.

Microdialysis Probe Implantation

Within 12 hrs after the 16th self-administration session, animals were briefly anesthetized with isoflurane, and implanted with a microdialysis probe through the previously implanted guide cannula. Each microdialysis probe was connected to a 1.0 ml gastight Hamilton 1000 series syringe mounted on a syringe pump (Razel®, Model A), and freshly prepared Ringer's solution was pumped through the probe. Animals implanted with the probe remained in a holding chamber overnight with the syringe pump speed set

at 0.261 $\mu\text{l}/\text{min}$. Bedding, food, and water were available in the holding chamber. One hour prior to the test session, the pump speed was changed to 1.63 $\mu\text{l}/\text{min}$.

Test Conditions

Animals were tested 24 hrs after the completion of training (at least 12 hrs post probe implantation) in one of six different testing conditions: Animals self-administered cocaine in the presence of ‘Certainty’, ‘Prediction Error’ and ‘Uncertainty’ cues. ‘Certainty’ cues were cues consistently associated with cocaine; ‘Prediction Error’ cues were cues consistently associated with saline; ‘Uncertainty’ cues were associated equally with both cocaine and saline. Certainty and Prediction Error conditions had undergone ‘Certainty’ cue-associative training and the ‘Uncertainty’ condition had undergone ‘Uncertainty 1’ or ‘Uncertainty 2’ cue-associative training. Similarly, animals also self-administered saline (non-reward) in the presence of ‘Certainty’, ‘Prediction Error’ and ‘Uncertainty’ cues. In this case, ‘Certainty’ cues were cues consistently associated with saline; ‘Prediction Error’ cues were cues consistently associated with cocaine; ‘Uncertainty’ cues were cues associated equally associated with both cocaine and saline.

Dialysis Test Session

Animals were placed in the operant chamber with the lever retracted for the first 30 min (Baseline). After 30 min., the house light illuminated, the lever extended into the chamber and the assigned cues were introduced into the chamber. Animals were allowed to respond once on the lever and received either a single self-administered injection of cocaine (1.5 mg/kg/infusion) or saline (approx 0.1 ml) infused over a 6-sec interval. The lever was then retracted for the remainder of the session. *In vivo* microdialysis samples were collected at 10 minute intervals across the entire test session, comprising three 10-minute baseline and three 10 minute test (e.g., post-cocaine or saline injection) samples. Locomotor activity units (photobeam breakages) were assessed in correspondence with dialysis sampling.

Assay of dialysate

The dialysates were analyzed for DA concentration using HPLC and electrochemical detection. Sample volume of 10 μ l was injected and its height was compared with 2 pg standard. The HPLC had a Shiseido Capcell C-18 narrow bore column, ESA model 5200 A Coulchem II detector, a Model 5041 cell and a Model 5020 Guard cell. The mobile phase composition was as follows- Sodium dihydrogen phosphate (75mM), Citric acid (4.76 mM), SDS 1 g/l, EDTA: 0.5mM, MeOH 8% and Acetonitrile 11%, pH 5.6). The analytical cell potential was set at + 200 mV (oxidation), guard cell potential was set at 400mV and the pump speed was set at 0.2 ml/min. The detection limit of DA was 0.05 pg/ μ l with a signal to noise ratio of 3:1. The amount of DA within each sample was determined by comparison with standards prepared and analyzed on the day of sample analysis. Data were collected and analyzed using an ESA Model 500 Data station.

Histological Analysis.

After the experiment, animals were euthanized by an overdose of pentobarbital sodium and brains were perfused using 0.9% saline and 10% formalin. The brains were carefully removed and stored in a 10% formaldehyde/30% sucrose solution. The probe placements within the NAcc, were verified from coronal sections (60 μ m) stained with crystal violet using the atlas of Paxinos and Watson (Paxinos and Watson, 1998).

Statistical Analyses

All data was analyzed using GB stat version 6.5 (Macintosh version). To compare the magnitude of NAcc DA responses between differently cued groups ('Certainty', 'Uncertainty' and 'Prediction Error'), data from animals receiving cocaine and saline during the test session were separately analyzed using two-way repeated measures ANOVAs (Cue Condition X Time). DA concentration in nM was corrected according to probe recovery rates and converted to percent of baseline for data analyses (overall baseline averaged from within-subject means of three baseline measurements).

Locomotor activity during the test session was also analyzed separately for cocaine and saline using two-way repeated measures ANOVA (Cue Condition X Time). Post hoc analyses (Fisher's LSD) were used to detect group or time differences (e.g., at least $p < 0.05$), when main and/or interaction effects were indicated by overall analyses.

2-4. Methods for Specific Aim 3

The methods for specific aim 3 were essentially the same as in specific aim 1 & 2. Following handling, food training and surgery animals underwent cue-associative training sessions.

Cue-Associative Training

Cue-associative training sessions were same as previously described except that animals were pretreated either with diazepam (0.25 mg/kg) (Hospira Inc. IL 60045, USA) or saline (control) prior to being introduced in the chamber. Just as in specific aim 1 there were two training groups: 'Certainty' and 'Uncertainty'. For the 'Certainty' cue-associative training, one visual/olfactory cue set was consistently associated with cocaine and another distinct set of cues was consistently associated with saline (non-reward). The 'Uncertainty' cue-associative training group had one set of cues associated with the first four cocaine sessions and another distinct set of cues associated with the first four saline sessions. For the last eight sessions, these cue/reinforcement sets were switched. Lever responses and locomotor activity during cocaine and saline self-administration sessions were the behavioral parameters that were measured.

Test conditions & Test session

Following 16 days cue-associative training, animals were implanted with a microdialysis probe and the same methodology as described for specific aim 2 was followed. No diazepam pretreatment was used on the test day. The groups were formed based on the pretreatment (diazepam vs. saline), cue-associative training

(‘Certainty’/‘Uncertainty’) and drug used on the test day (cocaine [1.5 mg/kg] or saline). Diazepam pretreated animals self-administering cocaine on the test day in the presence of ‘Certainty’, ‘Prediction Error’ and ‘Uncertainty’ cues were labeled as Certainty/Diazepam pretreatment, Prediction Error/Diazepam pretreatment and Uncertainty/Diazepam pretreatment respectively. ‘Certainty’ cues were cues consistently associated with cocaine, ‘Prediction Error’ cues were consistently associated with saline and ‘Uncertainty’ cues were cues equally associated with cocaine and saline. Similarly, control groups were labeled as Certainty/Saline pretreatment, Prediction Error/Saline pretreatment and Uncertainty/Saline pretreatment. If animal’s self-administered saline on the test day, they were classified as follows: ‘Certainty’ (when tested in the presence of saline associated cues), ‘Prediction Error’ (when tested in the presence of cocaine-associated cues) and ‘Uncertainty’ (when tested in the presence of cues equally associated with cocaine and saline). Accordingly, we had the following groups: Certainty/Diazepam pretreatment, Prediction Error/Diazepam pretreatment and Uncertainty/Diazepam pretreatment. Similarly, control groups were labeled as Certainty/Saline pretreatment, Prediction Error/Saline pretreatment and Uncertainty/Saline pretreatment. The dialysis test session, analysis of the dialysate and histological assessment was all done as described for specific aim 2.

Statistical Analysis

All data was analyzed using GB stat version 6.5 (Macintosh version). Lever responses across training days in the ‘Certainty’ and ‘Uncertainty’ cue-associative training groups were compared separately for cocaine and saline using three-way repeated measures ANOVAs (Pretreatment X Training X Session Days). Pretreatment was diazepam or saline (control) and Training was either ‘Certainty’ or ‘Uncertainty’. Similarly, locomotor activity across training days in the ‘Certainty’ and ‘Uncertainty’ cue-associative training groups was also analyzed separately for cocaine and saline using three-way repeated measure ANOVAs. To compare the magnitude of NAcc DA responses between

differently pretreated and cued groups ('Certainty', 'Uncertainty' and 'Prediction Error'), data from animals receiving cocaine and saline during the test session were separately analyzed using three-way repeated measures ANOVAs (Pretreatment X Cue Condition X Time). DA concentration in nM was corrected according to probe recovery rates and converted to percent of baseline for data analyses (overall baseline averaged from within-subject means of three baseline measurements). Locomotor activity during the dialysis test session was also analyzed separately for cocaine and saline using three-way repeated measures ANOVAs (Pretreatment X cue Condition X Time). Post hoc analyses (Fisher's LSD) were used to detect Pretreatment, Cue Condition or Time differences (e.g., at least $p < 0.05$) when main and/or interaction effects were indicated by overall analyses.

Odor Recognition Task

The olfactory recognition task was done to see if diazepam pretreatment (0.25 mg/kg) blocked the acquisition of olfactory memory. The task is based on the rationale that rats prefer novel odors as compared to known odors. The task was done in collaboration with Dr. T. Schallert's laboratory at University of Texas at Austin. The methods for this test have been described in a poster presented at the society of neuroscience conference in 2006 (Spinetta et al., 2006).

Method used for this dissertation

Sprague Dawley rats underwent intravenous catheterization (as described in the general methods section) after being handled for two weeks. Following surgery, animals were housed singly in polypropylene cages. The odor recognition task was divided into three phases carried over three days namely familiarization phase (day1), habituation test phase (day 2) and odor recognition test phase (day3).

Familiarization Phase (Day1)

48 hours before odor recognition test, animals were placed in an isolated room with a reversed light cycle. Four round wooden beads (25mm diameter) (Craftsworks .com) were placed in each cage of the rats to be tested. The beads absorb the odors of the animals. Beads placed in the cages of rats to be tested served as familiar odors and were called familiar beads. Beads were also placed in two other donor cages labeled as donor # 1 (2 beads for each rats to be tested) and donor # 2 (one bead for each rat to be tested). Each donor cage had 1-3 rats. Animals were left undisturbed for 24 hrs to familiarize with surroundings.

Habituation Phase (Day 2)

On the following day (day2), 1 hour before the habituation test was conducted, the beads were removed from the cages of donor # 1 and the rats to be tested. Beads were placed in sealable plastic bags. Animals were divided into two groups. One group received saline pretreatment and the other group received diazepam pretreatment (0.25 mg/kg). The pretreatments were carried out half-hour prior to the habituation test. Half- hour was used, as this was the same time we pretreated the animals prior to cue-associative training session. At the end of half-hour following pretreatment, habituation test was carried out. Water and food were removed to homogenize surrounding and reduce distractions. Habituation test consisted of three trials of 1 minute each with 1 minute of intertribal interval. During each trial, three beads belonging to the rat being tested (familiar beads) and one novel odor bead from donor # 1 were placed in random order in a row along the short side of cage. Rats were timed for one minute, starting from when they directly sniffed a bead. Timing stopped at one minute. If the animal was in the middle of a smell bout, the bout was allowed to be completed. During the one-minute intertrial interval, all beads were removed from the cage. Rat activities were recorded on video and analyzed at a later time point. During habituation, on the first trial the rats should devote more time on bead from donor # 1 as compared to the familiar beads, since it is a new odor. On the subsequent two trials, the rats will devote relatively less time on the bead from donor # 1

as compared to trial 1. The decrease in time spent over bead from donor # 1 over the three trials indicates habituation. After the third trial, the beads from donor # 1 used for the habituation test were discarded. The four familiar beads were put back in the cages of the rats that were tested. Food and water was returned, and animals were left untouched till the next morning.

Odor Recognition Test (Day 3)

On day 3, beads from rat cages were once again removed one hour prior to odor recognition test. Food and water were removed just prior to testing. During the test, four beads were placed in the cages of the rats being tested. One of the beads was obtained from donor # 2 (novel), one bead was obtained from donor # 1 (the rat showed habituation to bead from this cage the previous day) and the remaining two were familiar beads belonging to the rat being tested. Recognition test consisted of a single trial of 1 minute. The activity of the animal was recorded using a video camera to be analyzed at a later time. During analysis the time spent by the animal on each bead was recorded. Only time spent sniffing was recorded, time spent biting, gnawing, chewing, or playing (pushing or carrying beads with mouth) with the beads was not included in the analysis. During the olfactory recognition test, the rats will prefer to spend more time on the new bead (from donor # 2) as compared to bead from donor # 1, since they have been exposed to odor from donor # 1 on the previous day during habituation.

We used this test to see two things:

- 1) Does intravenous diazepam (0.25 mg/kg) pretreatment prior to habituation test affect habituation in the animals?
- 2) Does intravenous diazepam (0.25 mg/kg) pretreatment prior to the habituation test affect acquisition of olfactory memory and performance during the olfactory recognition test?

If diazepam pretreatment affected habituation, rats would not show a significant decrease in time devoted on bead from donor # 1 over the three one minute trials. On the other hand, if diazepam pretreatment affected acquisition of olfactory memory, then during the olfactory recognition test, animals would devote equal amount of time on beads from donor # 1 and donor # 2. See results on page 85

Statistical Analyses

During the habituation trials, time (seconds) spent on each bead during each trial was recorded. Data was analyzed using two-way repeated measures ANOVA (Bead X Trial). For the odor recognition test, data for each bead was expressed as percentage of total exploration time. Data was analyzed using one-way ANOVA. Post hoc test (Fisher's LSD) was used when main effects were indicated.

Chapter 3: Results

3-1. Results for Specific Aim 1

Effects of ‘Certainty’ and ‘Uncertainty’ cue-associative training on cocaine and saline self-administration

Three-factor repeated measures ANOVA (Training X Drug X Session Days) showed no significant overall Training, Drug or Training X Drug interaction effects [$F(2,104)=0.63$; $F(1,104)=0.75$; $F(2,104)=0.42$, respectively, all n.s.]. However, significant Session Days [$F(7,728)=12.42$; $p<0.0001$], Training X Session Days [$F(14,728)=3.84$; $p<0.0001$] and Training X Drug X Session Days [$F(14,728)=2.01$; $p=0.015$] interactions were observed. Post hoc analyses (Fishers’ LSD) showed that for ‘Certainty’ trained animals, no significant differences between response rates during matched-order cocaine and saline self-administration sessions occurred until the last session of each, when responses for cocaine were significantly ($p<0.05$) higher than for saline (See Fig.1). Animals trained under ‘Uncertainty’ conditions (‘Uncertainty 1’ and ‘Uncertainty 2’) elicited comparable response rates for cocaine and saline throughout all sessions (See Fig. 2A and 2B).

Effects of ‘Certainty’ and ‘Uncertainty’ cue-associative training on locomotor activity during cocaine and saline self-administration

A three-factor repeated measures ANOVA (Training X Drug X Session Days) showed significant Training [$F(2,104)=7.83$; $p=0.0007$], Drug [$F(1,104)=117.01$; $p<0.0001$], Session Days [$F(7,728)=30.31$; $p<0.0001$], and significant interaction effects [Training X Session Days, $F(14,728)=2.99$; $p=0.0002$; Drug X Session Days, $F(7,728)=23.62$; $p<0.0001$; Training X Drug X Session Days, $F(14,728)=2.05$, $p=0.013$]. Post hoc tests (Fisher’s LSD) revealed that cocaine- and non-stimulated locomotor activity were significantly greater in the ‘Uncertainty 1’ compared to most ‘Certainty’ trained operant sessions and some ‘Uncertainty 2’ sessions. During cocaine sessions, activity levels in the

'Uncertainty 1' group were significantly greater than the 'Certainty' group on days 5 ($p<0.05$), 7 ($p < 0.01$), 9 ($p<0.01$) and 13 ($p<0.05$). Similarly, during saline sessions activity levels in the 'Uncertainty 1' group were significantly greater than the 'Certainty' group on days 6 ($p<0.05$), 10 ($p<0.01$), 12 ($p<0.01$), 14 ($p<0.01$) and 16 ($p<0.01$). Activity levels in the 'Uncertainty 2' group were also higher than 'Certainty' group during a few cocaine [days 11 and 15 (both $p<0.05$)] and saline sessions [days 10, 12 and 16 (all $p<0.05$)]. Also, activity levels were higher in the 'Uncertainty 1' group as compared to the 'Uncertainty 2' group during a few cocaine [days 7 and 9 (both $p<0.01$)] and saline sessions [days 12 ($p<0.01$) and day 14 ($p<0.05$)] (see Fig. 3A and 3B).

Figure 1

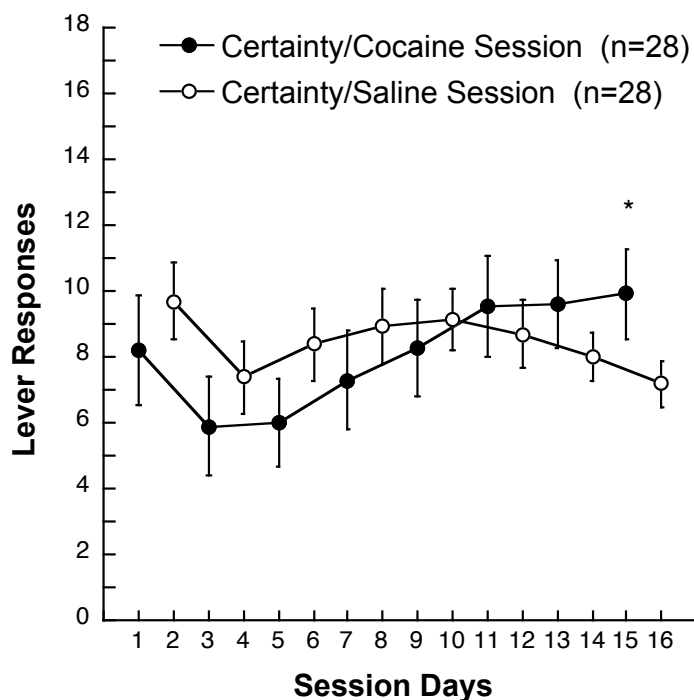


Fig. 1. Lever responses (mean \pm S.E.M.) for cocaine and saline in the ‘Certainty’ cue-associative training group

‘Certainty’ cue-associative training animals had one set of olfactory and visual cues consistently associated with cocaine and another distinct set of cues consistently associated with saline (non-reward). The rationale for this type of conditioning was to develop expectation of cocaine with the cocaine-associated cues and expectation of saline with the saline-associated cues. Animals showed a gradual increase in cocaine lever responses as the conditioning progressed. The lever responses for cocaine on Day 15 (last cocaine session) were significantly greater (* = $p < 0.05$) as compared to the lever responses for saline on Day 16 (last saline session).

Figure 2A

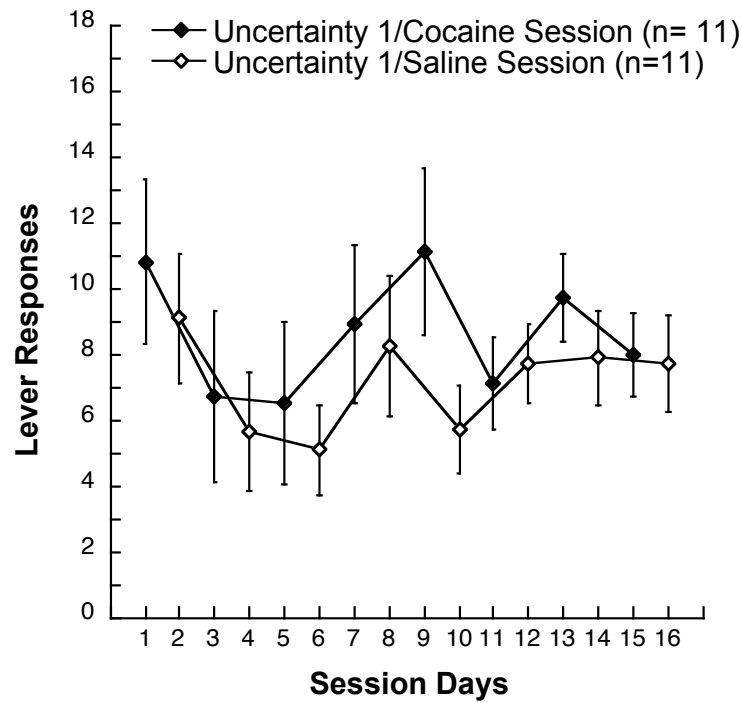


Fig. 2A. Lever responses (mean \pm S.E.M.) for cocaine and saline in the 'Uncertainty 1' cue-associative training group

In the 'Uncertainty 1' group cues were equally paired with both cocaine (0.5 mg/kg) and saline. The cues were interchanged between cocaine and saline on alternate days. As compared to the 'Certainty' group, animals from the 'Uncertainty 1' group did not develop preferential responding for cocaine.

Figure 2B

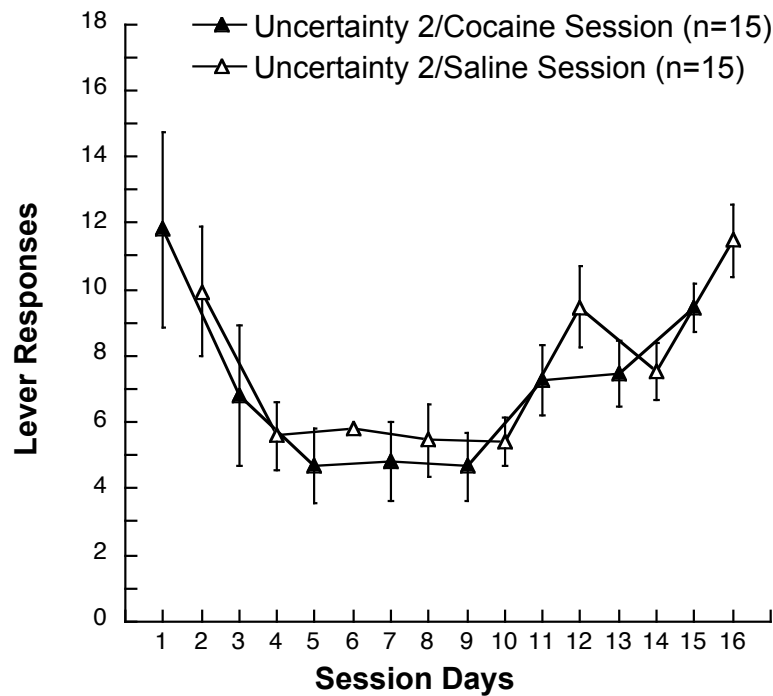


Fig. 2B. Lever responses (mean \pm S.E.M.) for cocaine and saline in the 'Uncertainty 2' cue-associative training group

In the 'Uncertainty 2' group also cues were equally paired with both cocaine (0.5 mg/kg) and saline. However, in the 'Uncertainty 2' group distinct cues were used for cocaine and saline for the first 8 sessions and then interchanged (see methods for details). In the 'Uncertainty 2' group also the animals did not develop preferential responding for cocaine.

Figure 3A

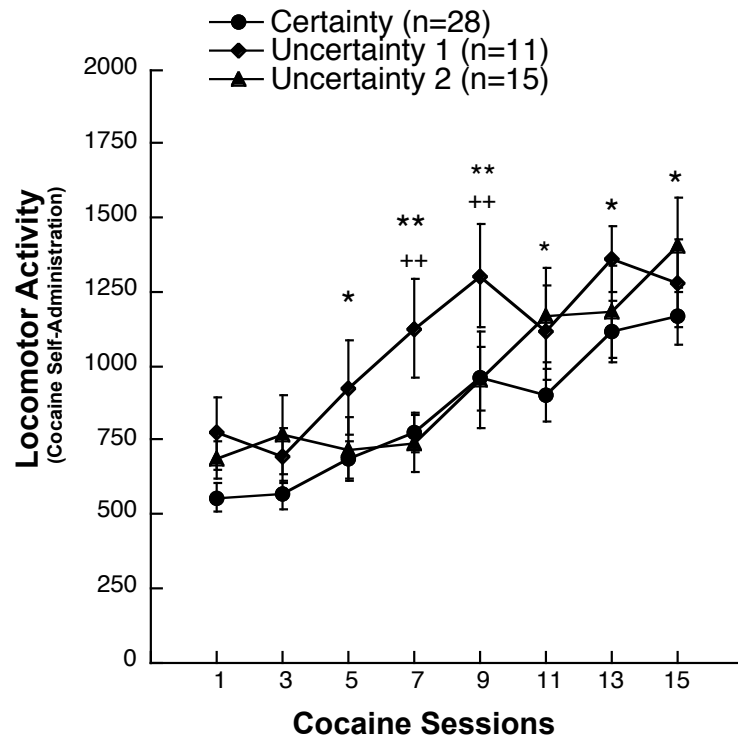


Fig. 3A. Locomotor activity (mean \pm SEM) during cocaine self-administration in the 'Certainty', 'Uncertainty 1' and 'Uncertainty 2' cue-associative training groups.

Locomotor activity in the 'Uncertainty 1' cue-associative training group was significantly greater (* $p < 0.05$; ** $p < 0.01$) as compared to the respective day in the 'Certainty' group. Locomotor activity in the 'Uncertainty 1' group was also significantly greater (++ $p < 0.01$) as compared to the respective day in the 'Uncertainty 2' group during cocaine self-administration.

Figure 3B

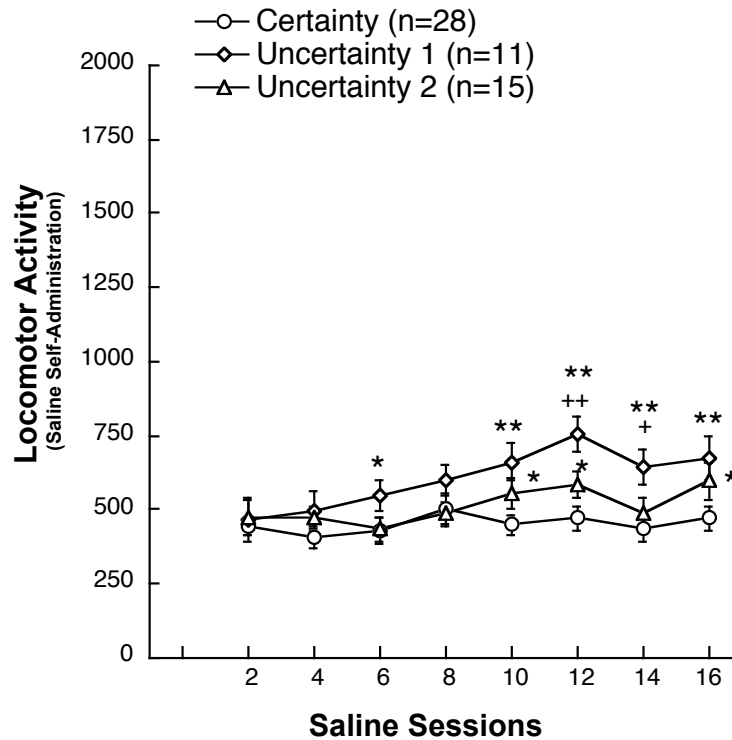


Fig. 3B. Locomotor activity (mean \pm SEM) during saline self-administration in the ‘Certainty’, ‘Uncertainty 1’ and ‘Uncertainty 2’ cue-associative training groups

Locomotor activity in both ‘Uncertainty’ groups was significantly greater (* $p < 0.05$, ** $p < 0.01$) as compared to the respective day in the ‘Certainty’ cue-associative training group during saline self-administration. Locomotor activity in the ‘Uncertainty 1’ group was significantly greater (+ $p < 0.05$; ++ $p < 0.01$) as compared to the respective day in the ‘Uncertainty 2’ group. The enhanced locomotor activity during saline self-administration in the uncertainty groups is important as it suggests that the animals are influenced by the cues during these sessions.

3-2. Results for Specific Aim 2

Nucleus accumbens dopamine (NAcc DA)

‘Uncertainty 1’ and ‘Uncertainty 2’ Groups

‘Uncertainty 1’ and ‘Uncertainty 2’ groups showed no significant differences in NAcc DA response to cocaine [$F(1,11)=0.036$; n.s.] or saline [$F(1,11)=2.24$; n.s.] or locomotor response to cocaine [$F(1,11)=0.47$; n.s.] or saline [$F(1,11)=0.85$, n.s.] during the test session. Therefore, data were combined into a single Uncertainty group for overall analyses of NAcc DA and locomotor activity. NAcc DA response to cocaine in the ‘Uncertainty’ groups is shown in Fig. 4.

NAcc DA: Effects of cocaine (1.5 mg/kg) self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues

A two-factor repeated measures ANOVA (Cue Condition X Time) showed significant Cue Condition [$F(2,22)=3.85$; $p=0.04$], Time [$F(5,110)=68.37$; $p<0.001$] and Cue Condition X Time interaction effects [$F(10,110)=3.99$; $p=0.0001$]. Post hoc analyses (Fisher’s LSD) revealed that cocaine-stimulated NAcc DA responses were significantly greater in the ‘Uncertainty’ group for the first two test intervals (e.g., 10 and 20 min post-injection) compared to both ‘Certainty’ and ‘Prediction Error’ groups (see Fig. 5). Cocaine-stimulated NAcc DA responses in the ‘Certainty’ and ‘Prediction Error’ conditions were comparable.

NAcc DA: Effects of saline self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues

A two-factor repeated measures ANOVA (Cue Condition X Time) showed no overall significant Cue Condition or Interaction effects [$F(2,26)=0.55$; and $F(10,130)=1.65$; both n.s.], but significant effects of Time [$F(2,26)=5.63$; $p=0.0002$]. Post hoc analyses

(Fisher's LSD) revealed that after cue presentation NAcc DA levels were significantly decreased in the presence of 'Prediction Error' cues as compared to all baseline measurements. NAcc DA levels showed gradual yet comparable reductions in NAcc DA levels in the presence of 'Certainty' and 'Uncertainty' cues across the testing interval (see Fig. 6).

Locomotor Activity: Effects of cocaine (1.5 mg/kg) self-administration in the presence of 'Certainty', 'Uncertainty' and 'Prediction Error' cues

Two-factor repeated measures ANOVA (Cue Condition X Time) showed no significant Cue Condition or Interaction effects [$F(2,21)=0.433$; $F(5,105)=0.87$, respectively, both n.s.], but significant effects of Time [$F(5,105)=22.59$; $P<0.0001$]. Posthoc analyses (Fisher's LSD) revealed significant increases in locomotor activity immediately following the self-administered cocaine injection in all groups (see Fig. 7).

Locomotor Activity: Effects of saline self-administration in the presence of 'Certainty', 'Uncertainty' and 'Prediction Error' cues

Two-factor repeated measures ANOVA (Cue Condition X Time) showed no significant Cue Condition effects [$F(2,21)=0.112$; n.s.], but significant Time and Interaction effects [$F(5,105)=6$; $P<0.0001$; $F(10, 105)=2.3$; $p=0.018$]. Post hoc analyses (Fisher's LSD) revealed that all groups decreased locomotor activity levels at comparable rates after eliciting a non-reinforced lever response (see Fig. 8). The locomotor activity in the presence of 'Certainty' cues was significantly lower in the last 10 minutes as compared to locomotor activity in the first 10 minutes immediately after saline self-administration.

**NAcc DA: Effect of cocaine (0.5 mg/kg) self-administration in the presence of
'Certainty', 'Uncertainty' and 'Prediction Error' cues**

A two-factor repeated measures ANOVA (Cue Condition X Time) showed no significant Cue Condition effects [$F(2,12)=0.04$; n.s.] and Interaction effects [$F(10,60)=0.14$;ns] but significant effect of time [$F(5, 10)=43.31$; $p=0.01$] (see Fig. 9).

Figure 4

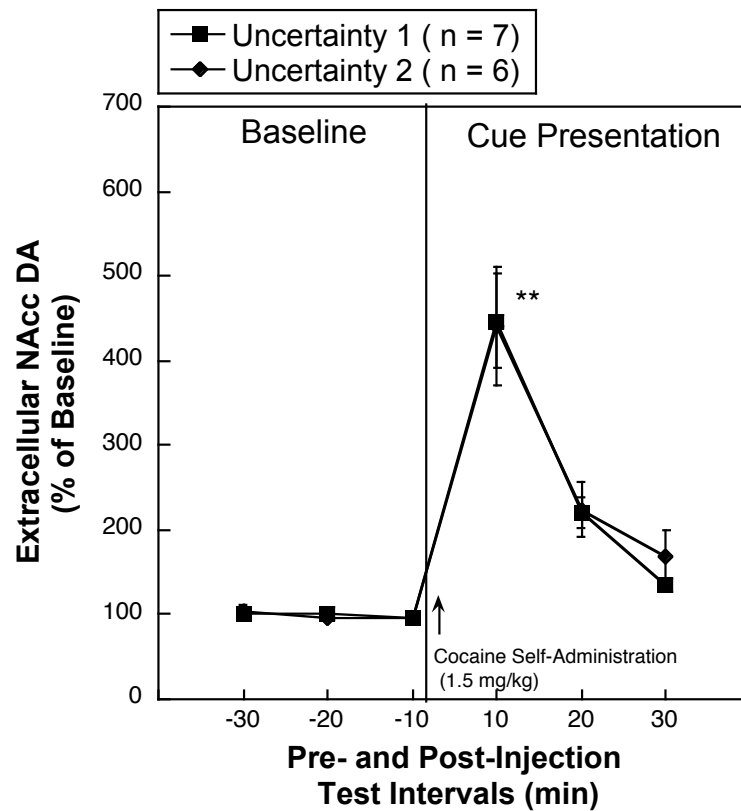


Fig. 4. Nucleus accumbens dopamine (NAcc DA) response to cocaine self-administration in the ‘Uncertainty 1’ and ‘Uncertainty 2’ Groups
Both groups showed significant increase (** $P < 0.01$) in NAcc DA over baseline following cocaine self-administration (1.5 mg/kg). Since there was no difference between the groups, from here on the data of the two groups is pooled and referred to as the ‘Uncertainty’ Group. The mean \pm SEM uncorrected DA nM levels in the ‘Uncertainty 1’ and ‘Uncertainty 2’ groups was 0.56 ± 0.17 and 0.6 ± 0.10 respectively.

Figure 5

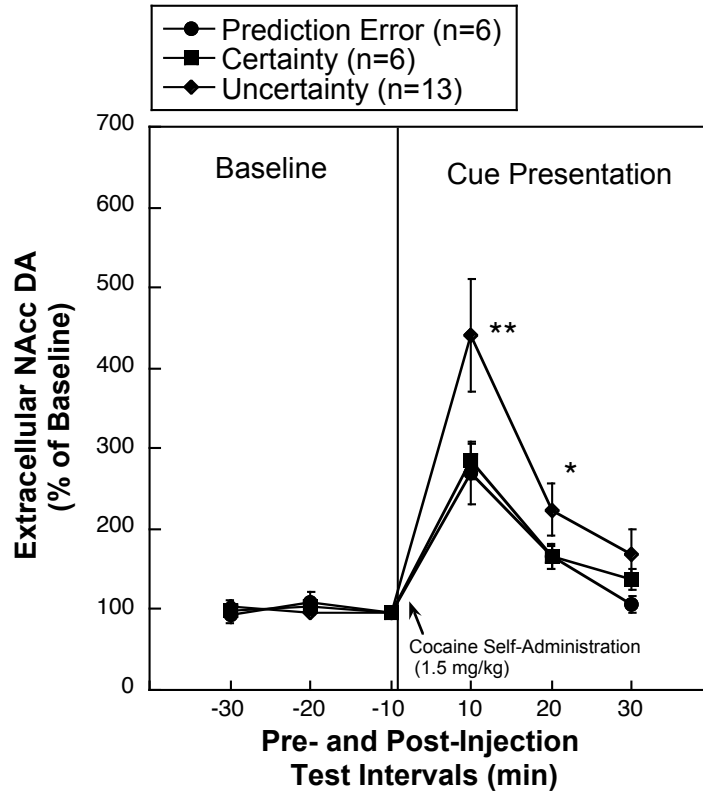


Fig. 5. Nucleus accumbens dopamine (NAcc DA) response to cocaine self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues

Following cocaine self-administration (1.5 mg/kg) NAcc DA was significantly greater (** $p < 0.01$; * $p < 0.05$) in the presence of ‘Uncertainty’ cues as compared to ‘Certainty’ and ‘Prediction Error’ cues. All three groups showed significantly greater enhancement in NAcc DA as compared to baseline following cocaine self-administration. [‘Certainty’ cues = cues consistently associated with cocaine, ‘Prediction Error’ cues = cues consistently associated with saline, ‘Uncertainty’ cues = cues equally associated with cocaine and saline]. ‘Certainty’ and ‘Prediction Error’ groups underwent ‘Certainty’ training and the ‘Uncertainty’ group underwent ‘Uncertainty’ training. The mean \pm SEM uncorrected DA nM levels in the ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ groups was 0.71 ± 0.16 , 0.58 ± 0.18 and 0.85 ± 0.26 respectively.

Figure 6

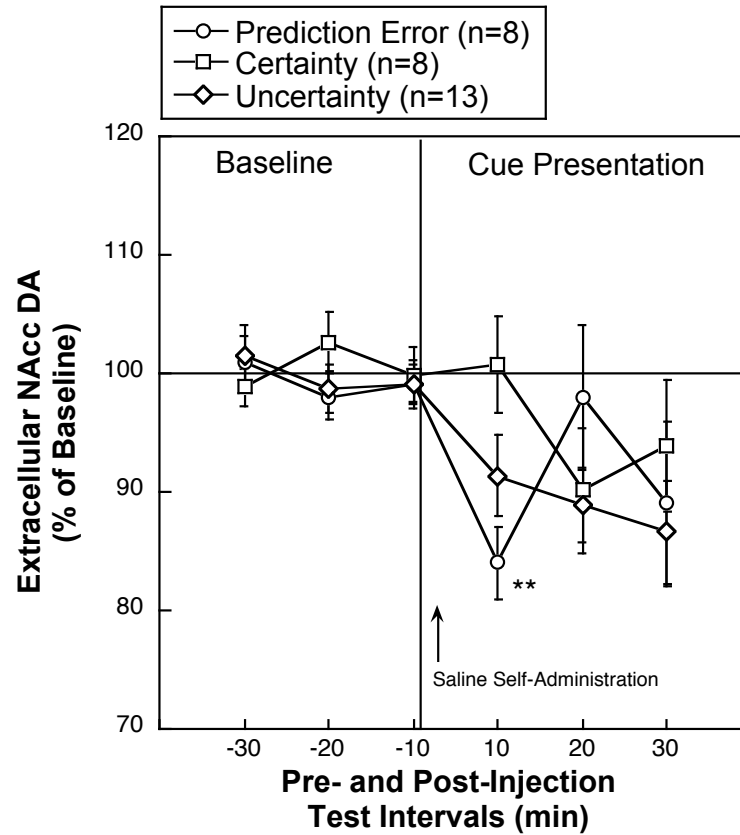


Fig. 6. Nucleus accumbens dopamine (NAcc DA) response to saline self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues

There was significant decrease from baseline (** $p < 0.01$) in the presence of ‘Prediction Error’ cues in the first 10 minutes immediately following saline self-administration [‘Certainty’ cues = cues consistently associated with saline, ‘Prediction Error’ cues = cues consistently associated with cocaine, ‘Uncertainty’ cues = cues equally associated with cocaine and saline]. The mean \pm SEM uncorrected nM levels in the ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ groups was 0.43 ± 0.06 , 0.62 ± 0.15 and 0.71 ± 0.13 respectively.

Figure 7

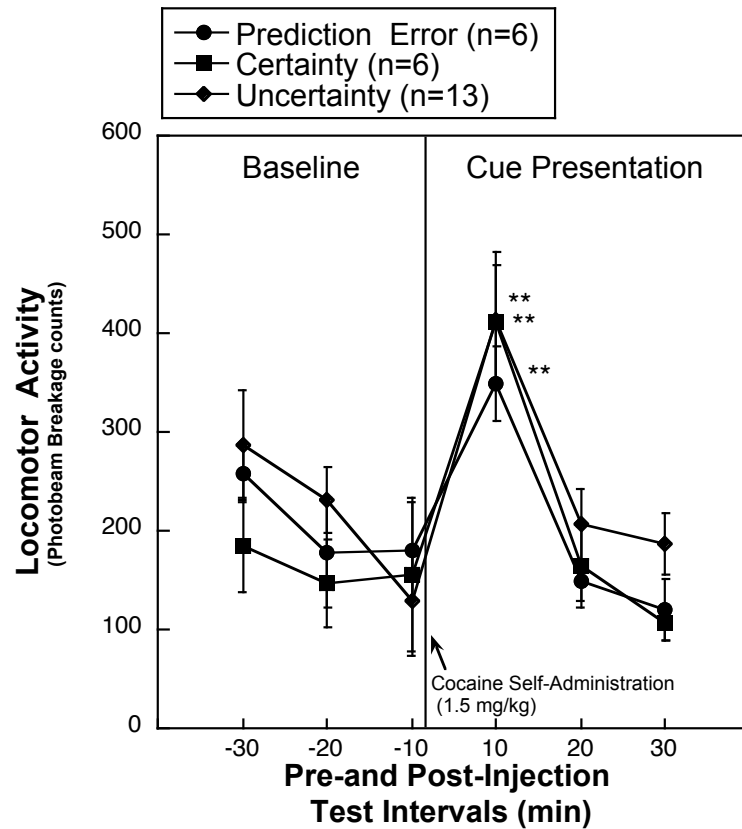


Fig. 7. Locomotor activity following cocaine self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues
Following cocaine self-administration (1.5 mg/kg) there was significant (** $p < 0.01$) increase in locomotor activity in all the three groups from their respective baseline activity at (-10) minutes. There was no significant difference between the groups following cocaine self-administration. Thus, there was a mismatch between NAcc DA and locomotor activity.

Figure 8

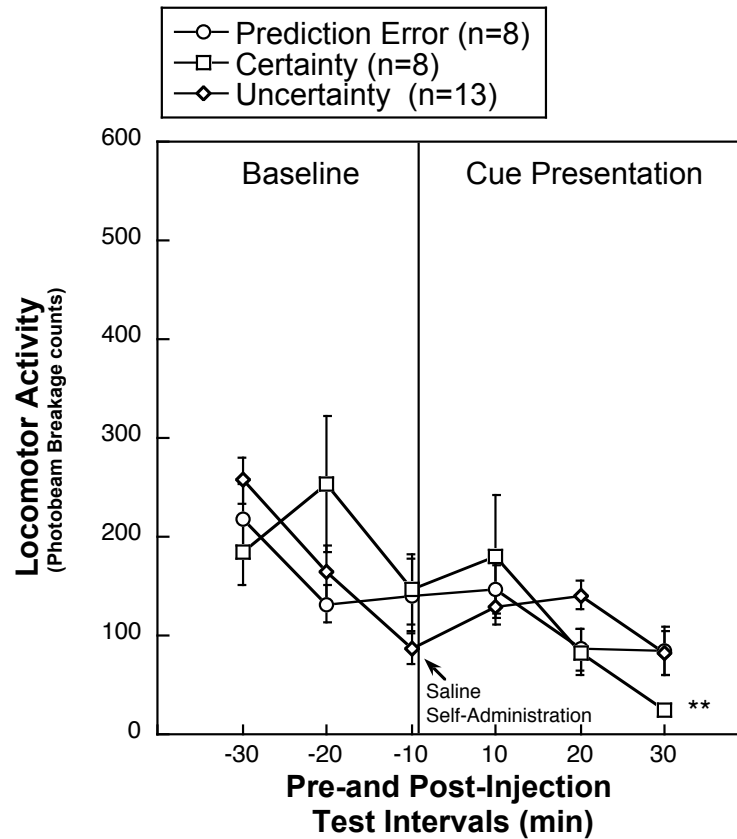


Fig. 8. Locomotor activity following saline self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues
Following saline self-administration there was significant decrease (** $p < 0.01$) in locomotor activity in the presence of ‘Certainty’ cues by the end of the dialysis session as compared to locomotor activity in the first 10 minutes after the lever response. No significant differences between the groups were observed.

Figure 9

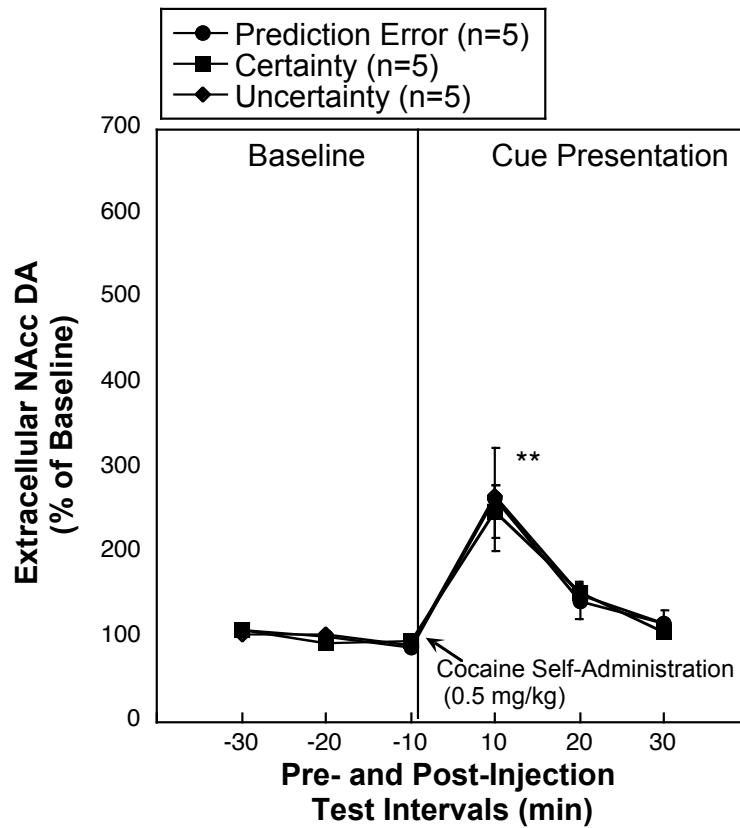


Fig. 9. Nucleus accumbens dopamine (NAcc DA) response to cocaine (0.5 mg/kg) self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues

Following cocaine self-administration (0.5 mg/kg) there was significant increase (** $p < 0.01$) in NAcc DA from baseline in all the three groups. There was no significant difference between the groups following self-administration of cocaine in the dose of 0.5 mg/kg. The mean \pm SEM uncorrected DA nM levels in the Certainty, Uncertainty and Prediction Error groups was 0.39 ± 0.06 , 0.48 ± 0.1 , 0.42 ± 0.08 respectively.

Figure 10

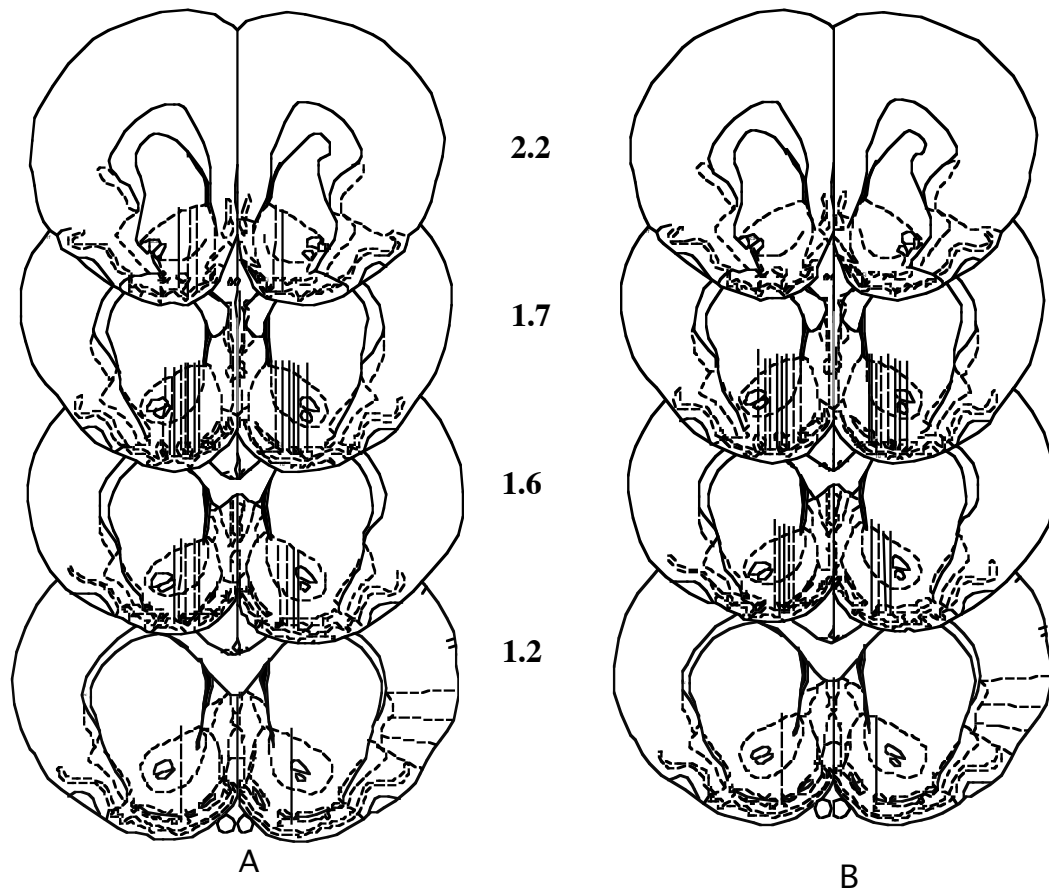


Fig. 10. Schematic representation of active dialysis probe membrane in the NAcc of animals who underwent (A) 'Certainty' and (B) 'Uncertainty' cue associative training

The numbers between the two figures indicate the distance of the slice in mm anterior to the bregma. The dotted lines represent the probes in animals tested with saline and the solid lines represent probes in animals tested with cocaine. The diagram was drawn from the atlas of Paxinos and Watson (Paxinos and Watson, 1998).

3-3. Results for Specific Aim 3

Lever Responses (self-administration) during cue-associative training

Effects of diazepam and saline pretreatment on cocaine self-administration in the ‘Certainty’ and ‘Uncertainty’ cue-associative training groups

Three-factor repeated measures ANOVA (Pretreatment X Training X Session Days) showed a significant overall Session Days effect [$F(7, 553) = 10.08, p < 0.01$]. There was a significant Pretreatment X Session Days [$F(7, 553) = 2.36; p = 0.02$] and Training X Session days interaction effect [$F(7, 553) = 4.48; p < 0.0001$]. No significant effects of Pretreatment or Training were seen [$F(1, 79) = 1.71; ns$; $F(1, 79) = 0.37; ns$]. In addition, there were also no significant Pretreatment X Training, or Pretreatment X Training X Session Days interactions observed. [$F(1, 79) = 0.57$; $F(7, 553) = 0.45$ respectively, all n.s.] (see Fig. 11A & 11B). Since there was a significant Pretreatment X Session Days interaction effect, Fisher's LSD was done to compare lever responses between diazepam and saline pretreated 'Certainty' cue-associative training groups. There was a significant enhancement in lever response for cocaine in the diazepam pretreated animals as compared to saline pretreated animals on day 9 ($p < 0.05$) and day 13 ($p < 0.01$) (see Fig. 11A)

Effects of diazepam and saline pretreatment on saline self-administration in the ‘Certainty’ and ‘Uncertainty’ cue-associative training groups

Three-factor repeated measures ANOVA (Pretreatment X Training X Session Days) showed a significant overall effect of Session Days [$F(7, 378) = 6.23, p < 0.01$]. No significant effects of Pretreatment or Training were seen [$F(1, 79) = 3.14$; $F(1, 79) = 1.75$; both ns]. In addition, there were also no significant Pretreatment X Training, Pretreatment X Session Days, Training X Session days, Pretreatment X Training X

Session Days interactions observed. [$F(1,79)=0.08$; $F(7,553)=1.43$; $F(7,553)=1.39$; $F(7,553)=1.38$ respectively, all n.s.] (see Fig. 12A & 12B).

Locomotor activity during cue-associative training

Effect of diazepam and saline pretreatment during cocaine self-administration in the ‘Certainty’ and ‘Uncertainty’ cue-associative training groups

Three-factor repeated measures ANOVA (Pretreatment X Training X Session Days) showed significant Pretreatment and Session Days effects [$F(1,79)=6.61$; $p<0.01$; $F(7,553)=50.04$; $p<0.0001$]. No significant effect of Training [$F(1,79)=1.18$; $p=0.27$] or Pretreatment X Training interaction was seen [$F(1,79)=0.05$; $p=0.8$]. Also, no significant Pretreatment X Session Days, Training X Session Days or Pretreatment X Training X Session Days interaction effects were observed [$F(7,553)=1.65$; $F(7,553)=1.08$; $F(7,553)=0.62$; all n.s.] (see Fig. 13).

Effect of diazepam and saline pretreatment during saline self-administration in the ‘Certainty’ and ‘Uncertainty’ cue-associative training groups

Three-factor repeated measures ANOVA (Pretreatment X Training X Session Days) showed significant Pretreatment, Training and Session Days main effects [$F(1,79)=8.08$; $p=0.005$; $F(1,79)=6.44$; $p=0.013$; $F(7,553)=9.37$; $p<0.0001$]. Significant Training X Session Days [$F(7,553)=4.55$; $p<0.0001$] and Pretreatment X Training X Session Days interaction effects [$F(7,553)=2.06$; $p=0.04$] were also seen. However, no significant Pretreatment X Training [$F(1,79)=0.013$; $p=0.9$] and Pretreatment X Session Days interaction effects were observed [$F(7,553)=1.03$; $p=0.4$] (see Fig. 14).

Figure 11A

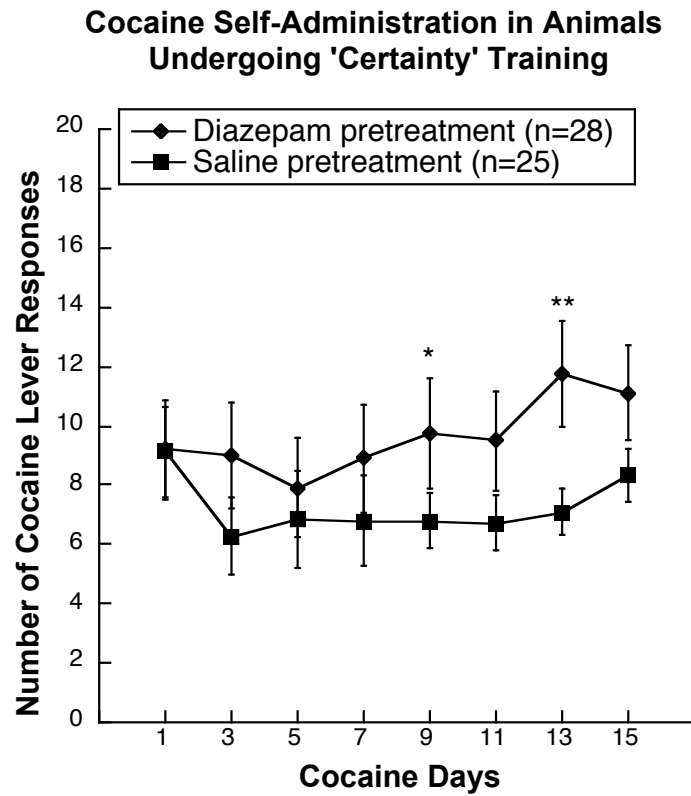


Fig. 11A. Lever responses (mean \pm S.E.M) for cocaine in animals undergoing 'Certainty' cue-associative training following diazepam and saline pretreatment

The diazepam pretreated animals showed significantly higher lever responses for cocaine as compared to saline pretreated animals on days 9 (* $p < 0.05$) and 13 (** $p < 0.01$).

Figure 11B

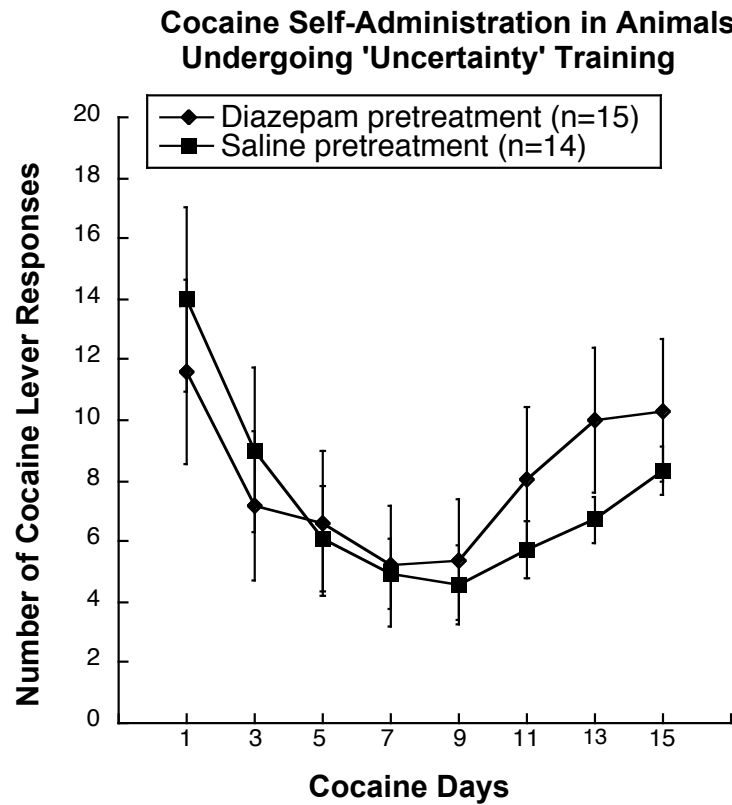


Fig. 11B. Lever responses (mean \pm S.E.M) for cocaine in animals undergoing 'Uncertainty' cue-associative training following diazepam and saline pretreatment

Diazepam pretreatment did not influence lever responses for cocaine in animals undergoing 'Uncertainty' cue-associative training.

Figure 12A

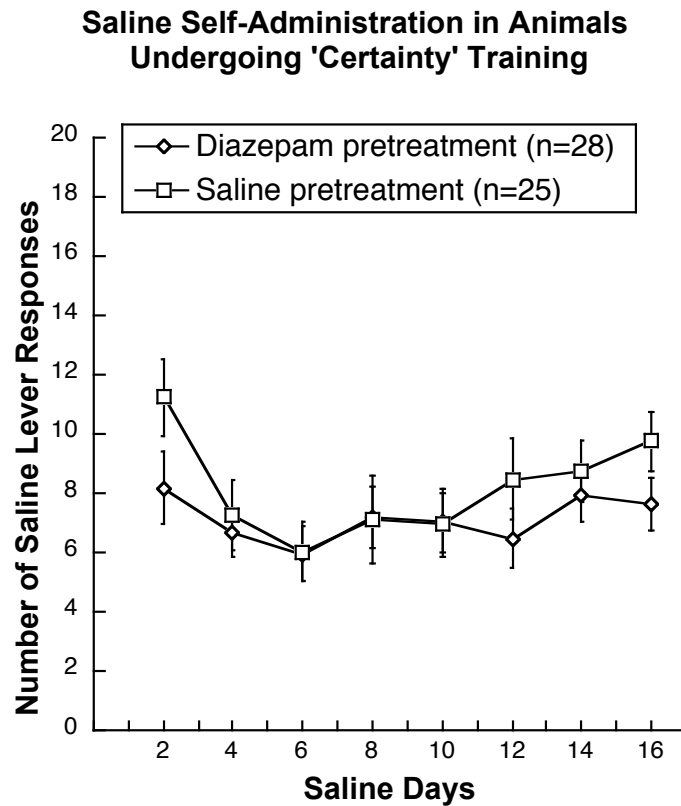


Fig. 12A. Lever responses (mean \pm S.E.M) for saline in animals undergoing 'Certainty' cue-associative training following diazepam and saline pretreatment

Diazepam pretreatment did not influence lever responses for saline in animals undergoing 'Certainty' cue-associative training.

Figure 12B

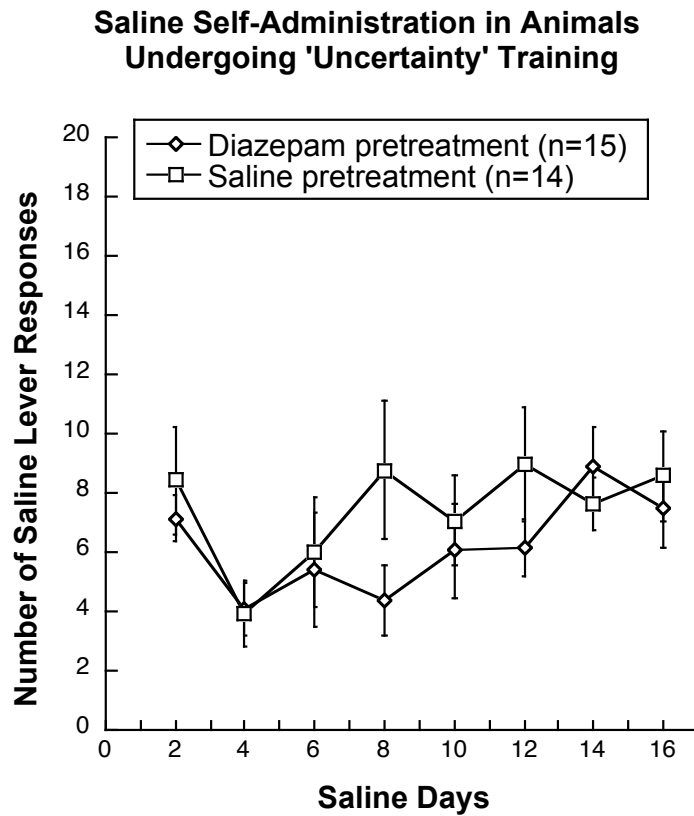


Fig. 12B. Lever responses (mean \pm S.E.M) for saline in animals undergoing 'Uncertainty' cue-associative training following diazepam and saline pretreatment

Diazepam pretreatment did not influence lever responses for saline in animals undergoing 'Uncertainty' cue-associative training.

Figure 13

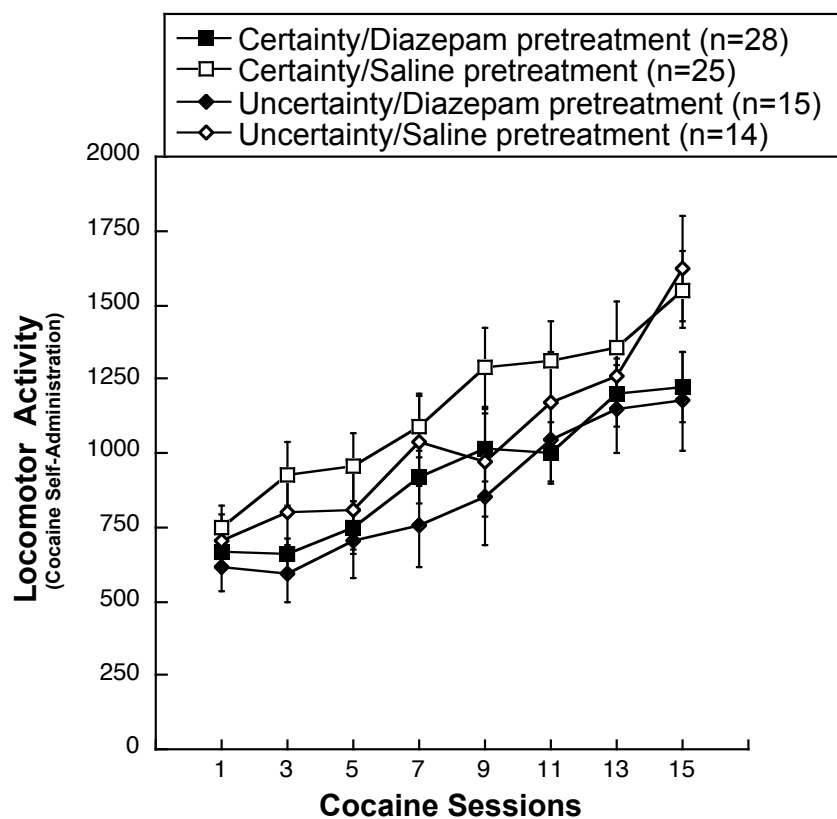


Fig.13. Locomotor activity (mean \pm SEM) during cocaine self-administration in animals undergoing ‘Certainty’ and ‘Uncertainty’ cue-associative training following diazepam and saline pretreatment
 No significant differences were seen between diazepam and saline pretreated animals in both ‘Certainty’ and ‘Uncertainty’ cue-associative training groups.

Figure 14

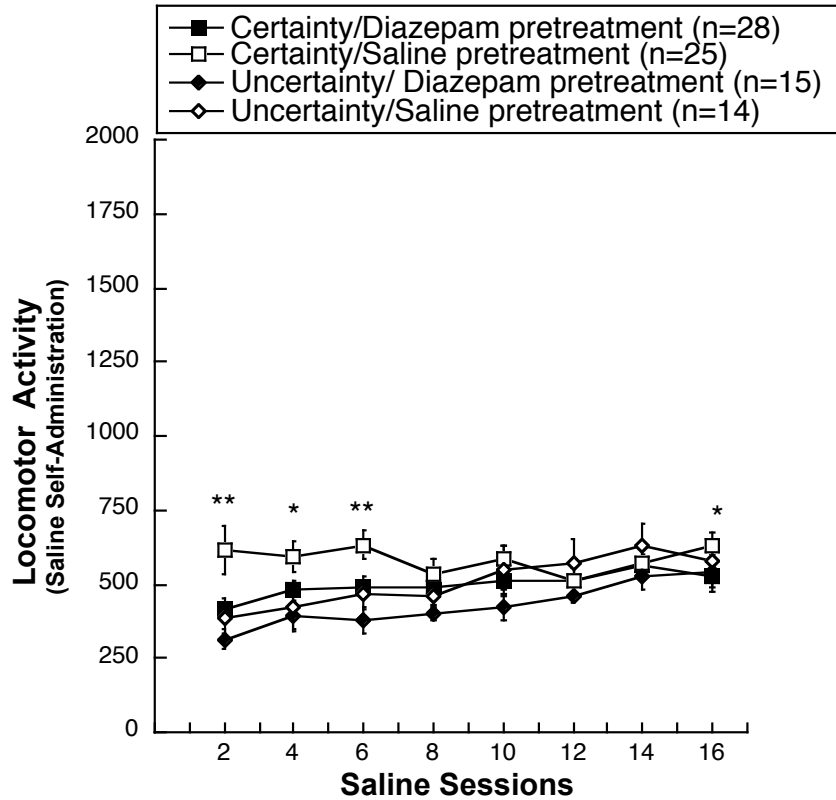


Fig.14. Locomotor activity (mean \pm SEM) during saline self-administration in animals undergoing ‘Certainty’ and ‘Uncertainty’ cue-associative training following diazepam and saline pretreatment

Saline pretreated animals undergoing ‘Certainty’ cue-associative training showed significantly higher locomotor activity during saline self-administration as compared to their diazepam pretreated counterparts on days 2, 4, 6 and 16. (** $p < 0.01$; * $p < 0.05$). No significant differences during saline self-administration were seen between diazepam and saline pretreated animals undergoing ‘Uncertainty’ cue-associative training.

Dialysis day data

Nucleus accumbens dopamine (NAcc DA)

Effect of cocaine self-administration on NAcc DA in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in diazepam and saline pretreated animals

Three factor repeated measures ANOVA (Pretreatment X Cue Condition X Time) showed a overall significant effect of Time [$F(5,170)=173.87$, $p<0.01$]. In addition, Cue Condition X Time and Pretreatment X Cue condition X Time interaction effects were observed [$F(10,170)=2.31$, $p<0.01$; $F(10,170)=1.89$, $p<0.05$ respectively]. There were no overall significant effects of Pretreatment or Cue Condition [$F(1,34)=1.41$; $F(2,34)=2.75$ respectively; both n.s.]. No significant interaction effects of Pretreatment X Cue condition or Pretreatment X Time were observed [$F(2,34)=2.85$; $F(5,170)=1.92$ respectively both n.s.]. Post hoc analyses (Fisher’s LSD) indicated that diazepam pretreated ‘Certainty’ ($p<0.01$) and ‘Prediction Error’ ($p<0.05$) groups showed a significantly greater enhancement in NAcc DA response following cocaine as compared to their respective saline pretreated groups. No significant difference was seen between diazepam pretreated ‘Certainty’ and ‘Uncertainty’ groups, while there was significant difference between saline pretreated ‘Certainty’ and ‘Uncertainty’ groups ($p<0.01$) (See Fig. 15A & 15B).

Effects of saline self-administration on NAcc DA in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in diazepam and saline pretreated animals

Three factor repeated measure ANOVA (Pretreatment X Cue Condition X Time) showed significant effect of Time and Pretreatment X Cue Condition X Time interaction effects

[F(5,180)=0.02, $p < 0.01$; F(10,180), $p < 0.05$) No significant Pretreatment or Cue Condition effects were seen [F(1,36)=0.60; F(2,36)=2.65 respectively, both n.s.]. Also, no significant Pretreatment X Cue Condition, Pretreatment X Time and Cue Condition X Time interaction effects were seen [F(2,36)=2.97; F(5,180)=1.37; F(10,180)=1.34 respectively, all n.s.]. Post-hoc analyses (Fisher's LSD) showed that the saline pretreated 'Prediction Error' group showed a significant decrease ($p < 0.05$) in NAcc DA from baseline (see Fig. 16A & 16B).

Locomotor activity

Effects of cocaine self-administration on locomotor activity in the presence of 'Certainty', 'Uncertainty' and 'Prediction Error' cues in diazepam and saline pretreated animals

A three factor repeated measure ANOVA (Pretreatment X Cue Condition X Time) showed a significant effect of Pretreatment [F(1,34)=4.01; $p < 0.05$] and Time [F(5,170)=55.38; $p < 0.01$] along with a significant Pretreatment X Time interaction effect [F(5,170)=4.62; $p < 0.01$]. No significant effect of Cue Condition [F(2,34)=1.68; n.s.] was seen. No significant Pretreatment X Cue Condition [F(2,34)=0.43, n.s.], Cue Condition X Time [F(10,170)=1.67, n.s.], Pretreatment X Cue Condition X Time [F(10,170)=1.00, n.s.] interaction effects were seen (see Fig. 17A & 17B).

Effects of saline self-administration on locomotor activity in the presence of 'Certainty', 'Uncertainty' and 'Prediction Error' cues in diazepam and saline pretreated animals

A three factor repeated measure ANOVA (Pretreatment X Cue Condition X Time) showed only significant effect of Time [F(5,180)=23.78; $p < 0.01$]. No Pretreatment [F(1,36)=0.05, n.s.] or Cue Condition [F(2,36)=0.03, n.s.] effect was seen. Neither any significant Pretreatment X Cue Condition [F(2,36)=0.83, n.s.], Pretreatment X Time

[F(5,10)=1.24, n.s.], Cue Condition X Time [F(10,180)=0.80, n.s.], Pretreatment X Cue Condition X Time [F(10,180)=1.29, n.s.] interaction effects were seen (see Fig. 18A & 18B).

Odor recognition task data

Effect of diazepam pretreatment on habituation trials

A two-way repeated measures ANOVA (Trial X Bead) showed significant effects of Trial [F(2,42)=8.90; $p=0.006$], Bead [F(3,6)= 72.86; $p<0.001$] and Trial X Bead interaction [F(6,126)=9.30; $p<0.001$]. Post hoc analyses (Fisher's LSD) revealed that the diazepam pretreated animals spent significantly greater time on bead from donor # 1 in trial 1 as compared to trial 2 and trial 3. They also spent significantly greater time on bead from donor # 1 in trial 2 as compared to trial 3. This means that diazepam pretreatment did not effect the habituation to the novel odor as the rats spent significantly less time on the new bead as the trials progressed (see Fig. 19A).

Effect of saline pretreatment on habituation trials

A two-way repeated measures ANOVA (Trial X Bead) showed significant effects of Trial [F(2,19)=15.38; $p=0.0001$], Bead [F(3,6)= 63.55; $p<0.0001$] and Trial X Bead interaction [F(6,57)=10.11; $p<0.001$]. Post hoc analyses (Fisher's LSD) revealed that the diazepam-pretreated animals spent significantly greater time on bead from donor # 1 in trial 1 as compared to trial 2 and trial 3. They also spent significantly greater time on bead from donor # 1 in trial 2 as compared to trial 3. This means that saline pretreatment did not effect the habituation to the novel odor, as the rats spent significantly less time on the new bead as the trials progressed (see Fig. 19B).

Odor recognition test data in animals pretreated with diazepam

A one-way ANOVA showed a significant effect [F(3,56)=66.94; $P<0.0001$]. Post hoc analyses revealed that diazepam pretreated animals spent significantly greater time

($p < 0.01$) on novel bead from donor # 2 as compared to any other bead. This means that diazepam pretreatment on the previous day did not affect the acquisition of olfactory memory in the rats (see Fig. 20A)

Odor recognition test data in animals pretreated with saline

A one-way ANOVA showed a significant effect [$F(3,28)=22.69$; $P < 0.0001$]. Post hoc analyses revealed that saline-pretreated animals spent significantly greater time ($p < 0.01$) on novel bead from donor # 2 as compared to any other bead. This means that saline pretreatment on the previous day did not affect the acquisition of olfactory memory in the rats (see Fig. 20B).

Figure 15 A

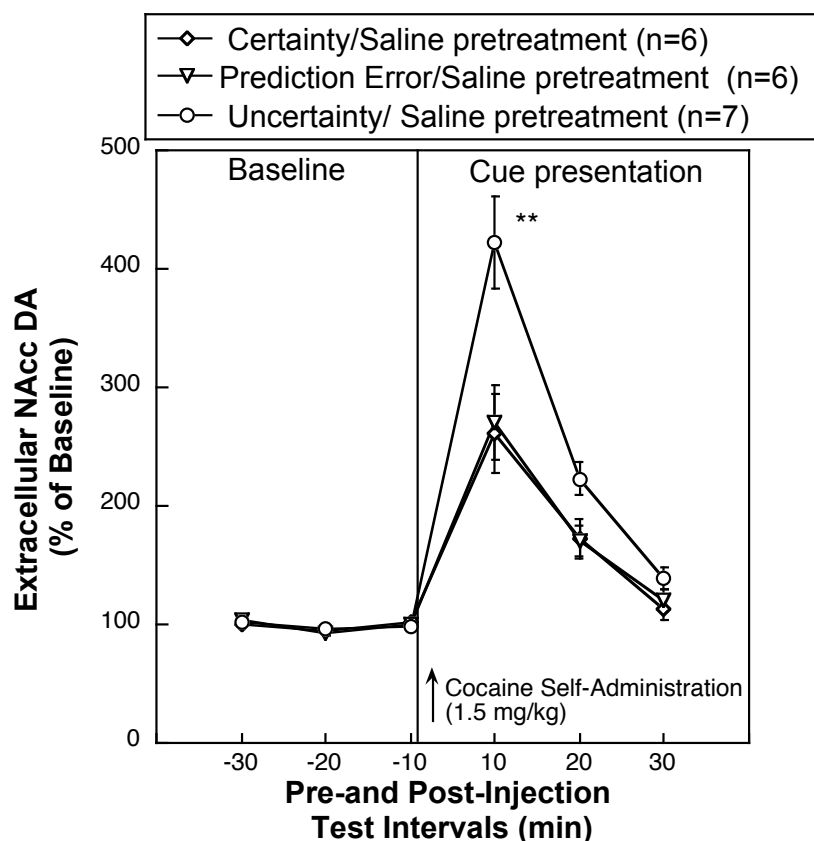


Fig. 15A. NAcc DA following cocaine self-administration in presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with saline during cue-associative training.

Animals pretreated with saline during conditioning, showed significant enhancement (** $p < 0.05$) in NAcc DA following cocaine self-administration the presence of ‘Uncertainty’ cues as compared to presence of ‘Certainty’ and ‘Prediction Error’ cues. Thus, we were able to successfully repeat our previous data (see Fig. 5). The mean \pm SEM uncorrected DA nM levels in the saline pretreated ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ groups was 1.1 ± 0.32 , 0.75 ± 0.07 and 0.81 ± 0.06 respectively.

Figure 15B

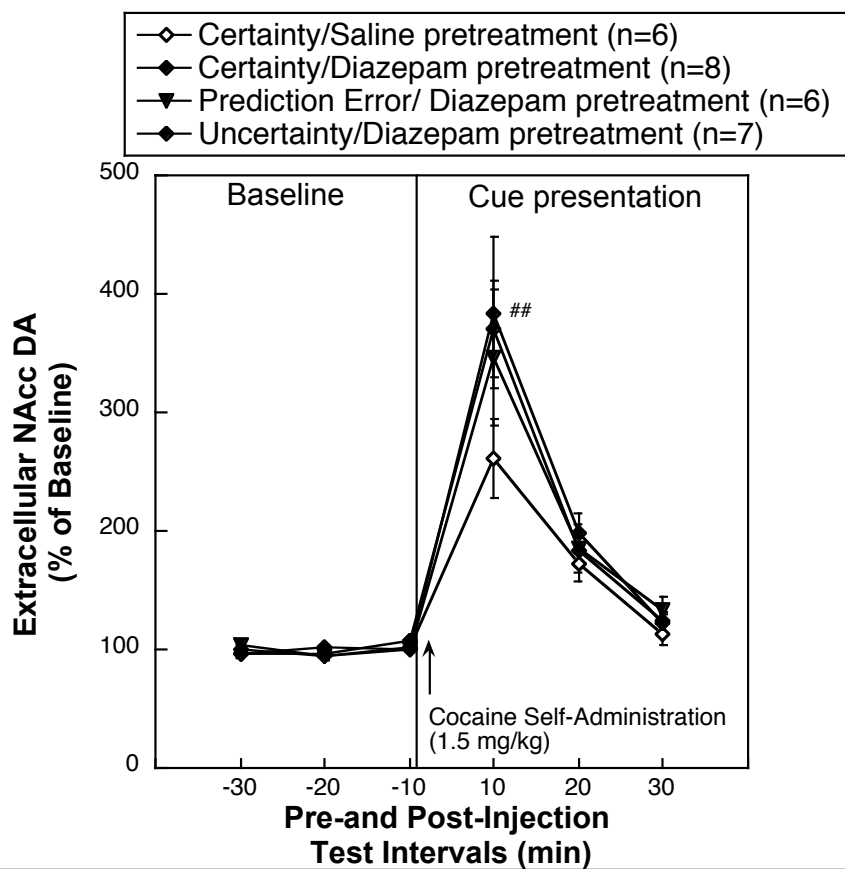


Fig. 15B. NAcc DA following cocaine self-administration in presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with diazepam during cue-associative training

This graph shows two things: first, animals pretreated with diazepam during conditioning did not show the differential cocaine-stimulated NAcc DA response in the presence of ‘Certainty’ and ‘Uncertainty’ cues as seen in saline pretreated animals (see Fig. 15A). Second, NAcc DA response to cocaine in the presence of ‘Certainty’ cues was significantly greater (## $p < 0.05$) in diazepam pretreated animals as compared to saline pretreated animals. The mean \pm SEM uncorrected DA nM levels in the diazepam pretreated Certainty, Uncertainty and Prediction Error groups was 0.64 ± 0.10 , 0.75 ± 0.11 and 0.88 ± 0.09 respectively.

Figure 16A

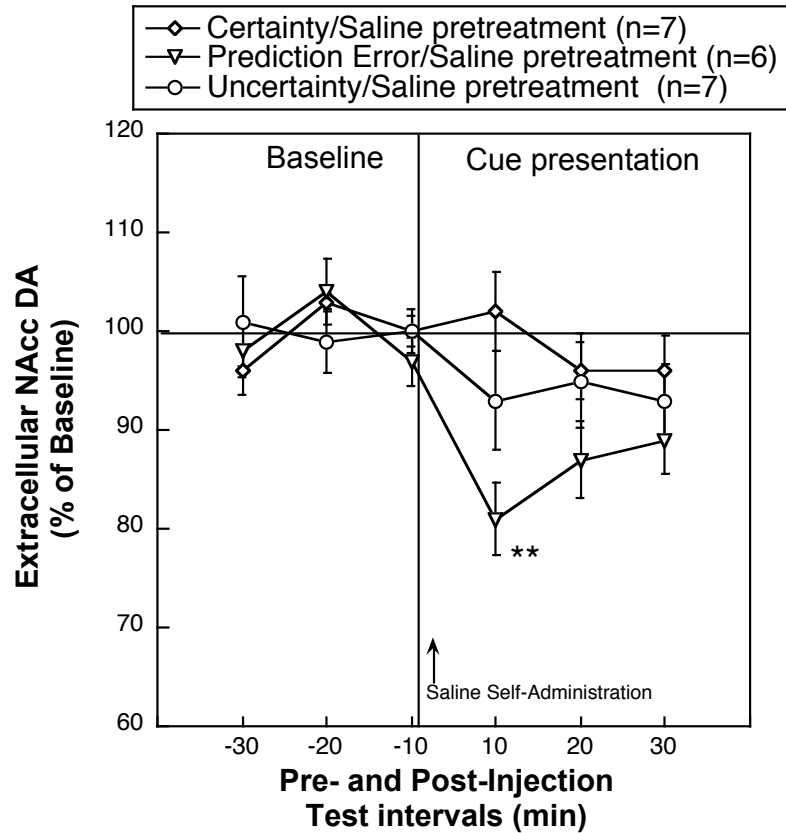


Fig. 16A. NAcc DA following saline self-administration in presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with saline during cue-associative training
In the presence of ‘Prediction Error’ cues, animals that received saline pretreatment during cue-associative training, showed a significant decrease from baseline in NAcc DA following saline self-administration (** $p < 0.01$). ‘Prediction Error’ cues were cues associated consistently with cocaine during cue-associative training. This is similar to our previous data (see fig. 6). The mean \pm SEM uncorrected DA nM levels in the saline pretreated ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ groups was 0.57 ± 0.11 , 0.54 ± 0.08 and 0.80 ± 0.14 respectively.

Figure 16B

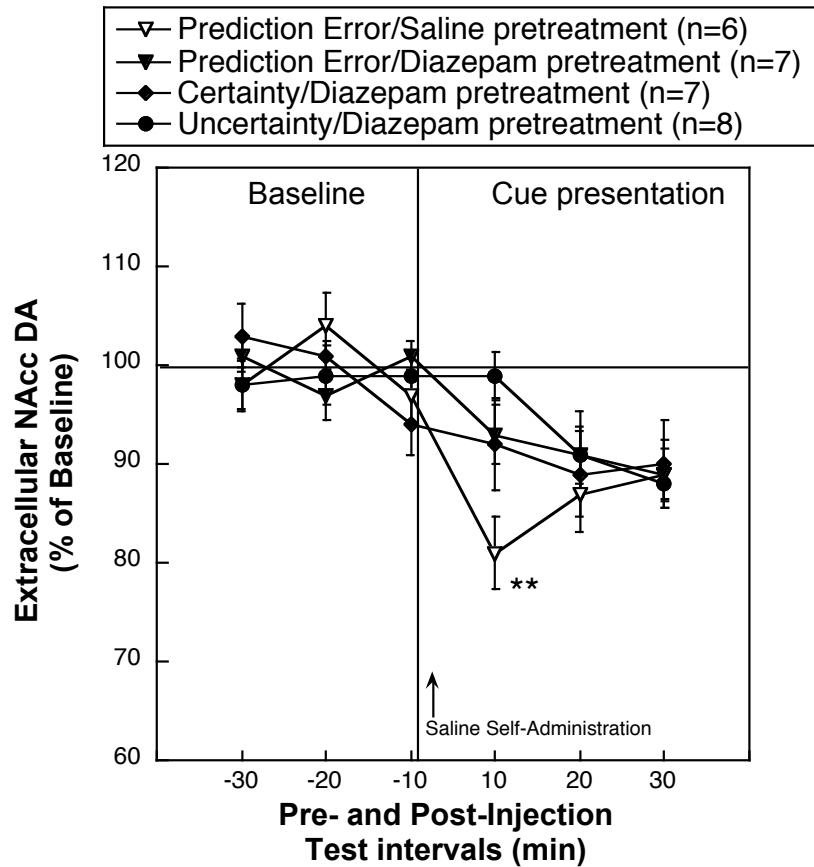


Fig. 16B. NAcc DA following saline self-administration in presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with diazepam during cue-associative training

Animals pretreated with diazepam during cue-associative training, did not show a significant decrease from baseline in NAcc DA following saline self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues. (** p<0.01 indicates a significant decrease from baseline in NAcc DA in saline pretreated animals in the presence of ‘Prediction Error’ cues). The mean \pm SEM uncorrected DA nM levels in the diazepam pretreated Certainty, Uncertainty and Prediction Error groups was 0.58 ± 0.05 , 0.63 ± 0.07 and 0.64 ± 0.10 respectively.

Figure 17A

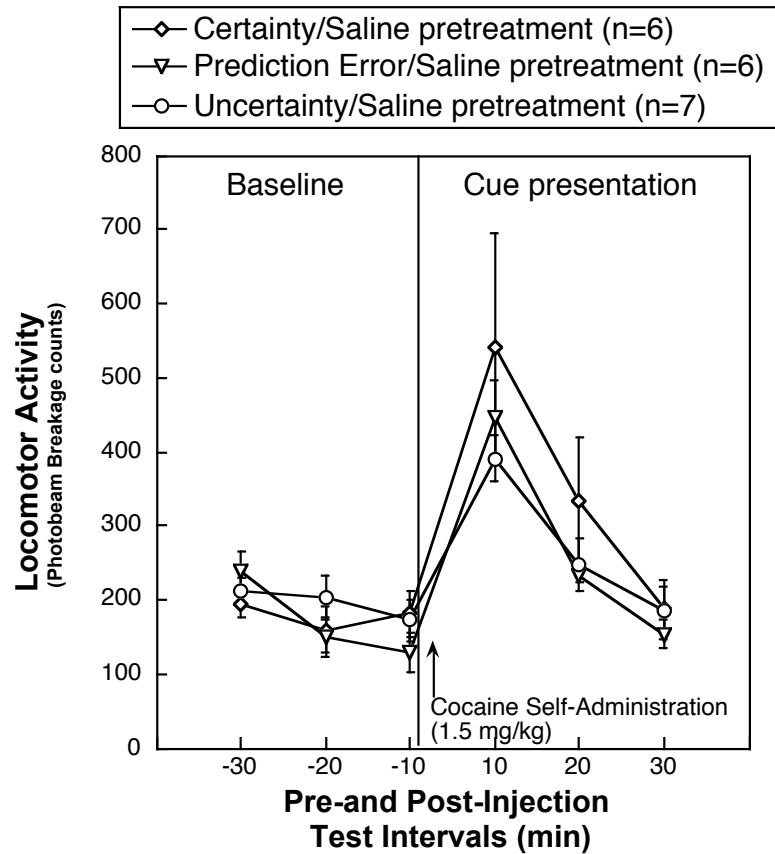


Fig. 17A. Locomotor activity following cocaine self-administration in presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with saline during cue-associative training
No significant difference in locomotor activity was seen following cocaine self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with saline during cue-associative training.

Figure 17B

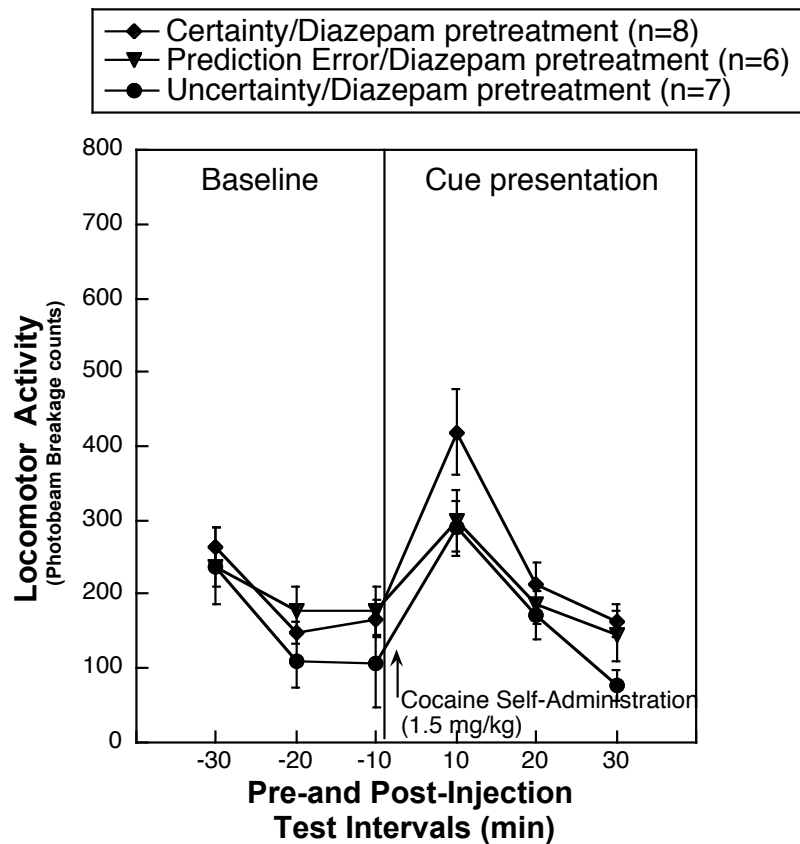


Fig. 17 B. Locomotor activity following cocaine self-administration in presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with diazepam during cue-associative training

No significant differences in locomotor activity were seen following cocaine self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with diazepam during cue-associative training.

Figure 18A

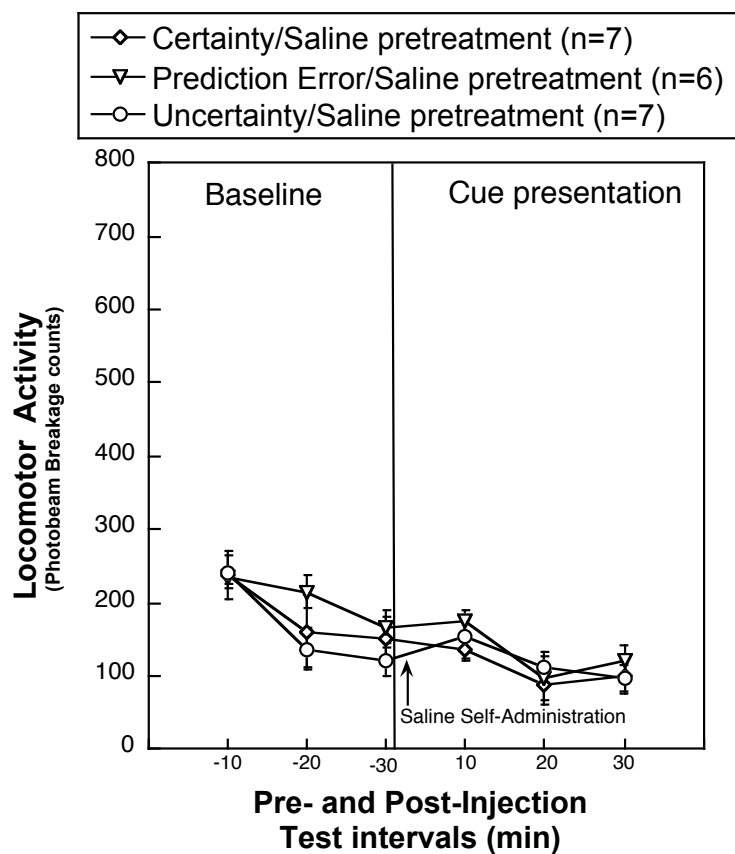


Fig. 18A. Locomotor activity following cocaine self-administration in presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with saline during cue-associative training

No significant differences in locomotor activity were seen following saline self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in animals pretreated with saline during cue-associative training.

Figure 18B

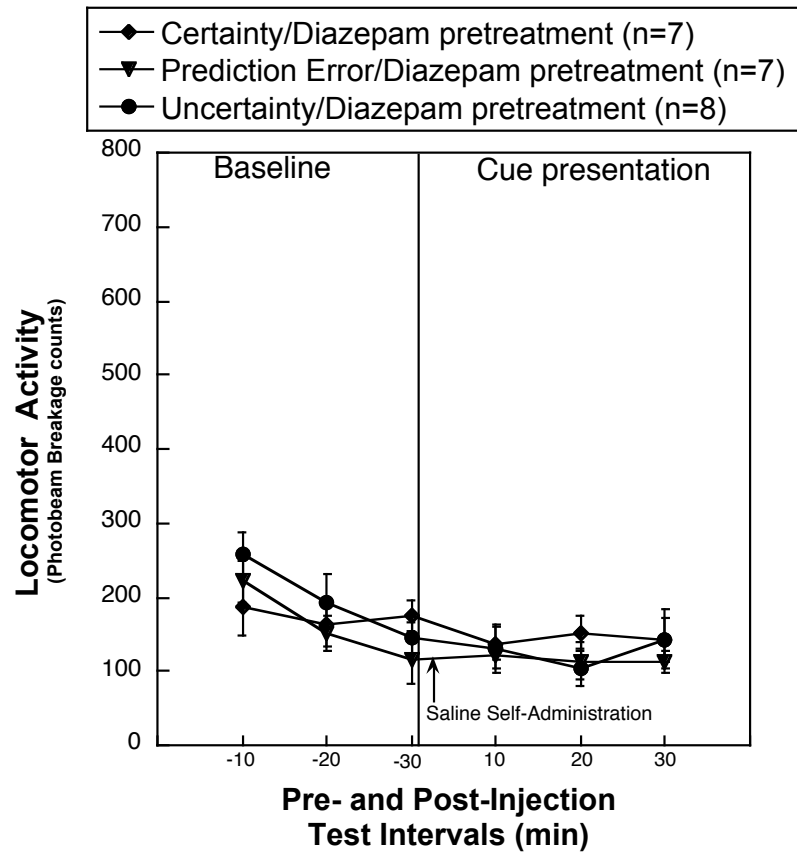


Fig. 18B. Locomotor activity following saline self-administration in presence of 'Certainty', 'Uncertainty' and 'Prediction Error' cues in animals pretreated with diazepam during cue-associative training
No significant differences in locomotor activity were seen following saline self-administration in the presence of 'Certainty', 'Uncertainty' and 'Prediction Error' cues in animals pretreated with diazepam during cue-associative training.

Figure 19

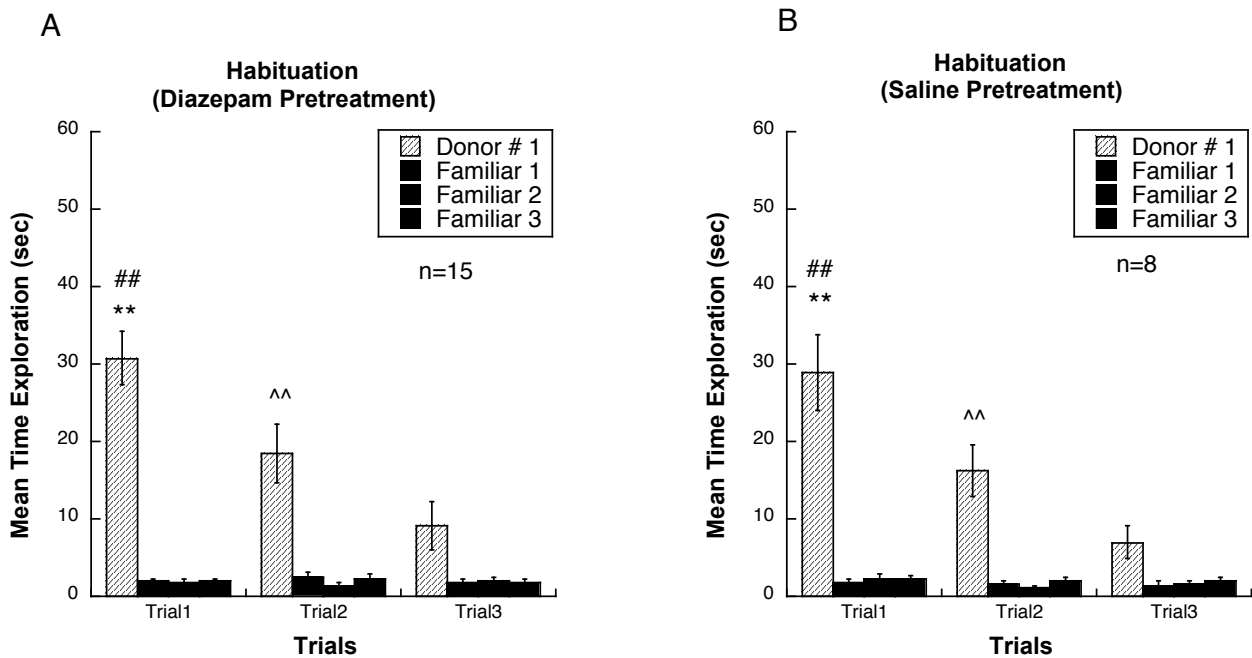


Fig. 19. Time spent (mean \pm S.E.M) on beads during habituation trials following (A) Diazepam (B) Saline pretreatment

The figure shows time spent by the animals on the new bead from donor # 1 as compared to familiar beads (beads from the animal's own cage) over three trials of one minute each. Over the three trials both, diazepam (0.25 mg/kg) and saline pretreated animals show habituation. In trial 1 both, diazepam and saline pretreated animals spent significantly greater amount of time on bead from donor # 1 as compared to trial 2 (** $p < 0.01$) and trial 3 (## $p < 0.01$). Time spent on bead from donor # 1 is significantly greater in trial 2 as compared to trial 3 (^ $p < 0.01$). This data suggests that diazepam pretreatment does not affect habituation.

Figure 20

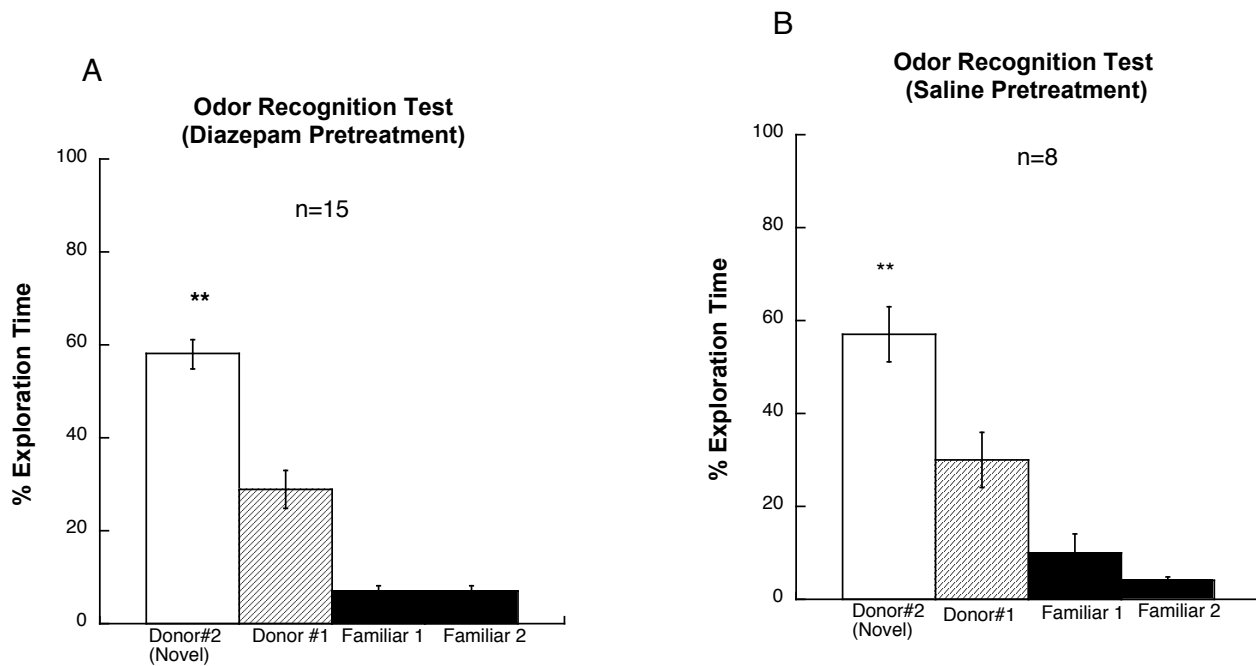


Fig. 20. Time spent on the beads during the odor recognition test in animals pretreated with (A) Diazepam and (B) Saline on the previous day

The olfactory memory test was a one minute trial. The data is expressed as percentage of time spent on all the beads. Both, diazepam (0.25 mg/kg) and saline pretreated animals spent significantly greater time (** $p < 0.01$) with the novel bead from donor # 2 as compared to bead from donor # 1. This data suggests that diazepam pretreatment on the previous day did not affect the acquisition of olfactory memory.

Figure 21

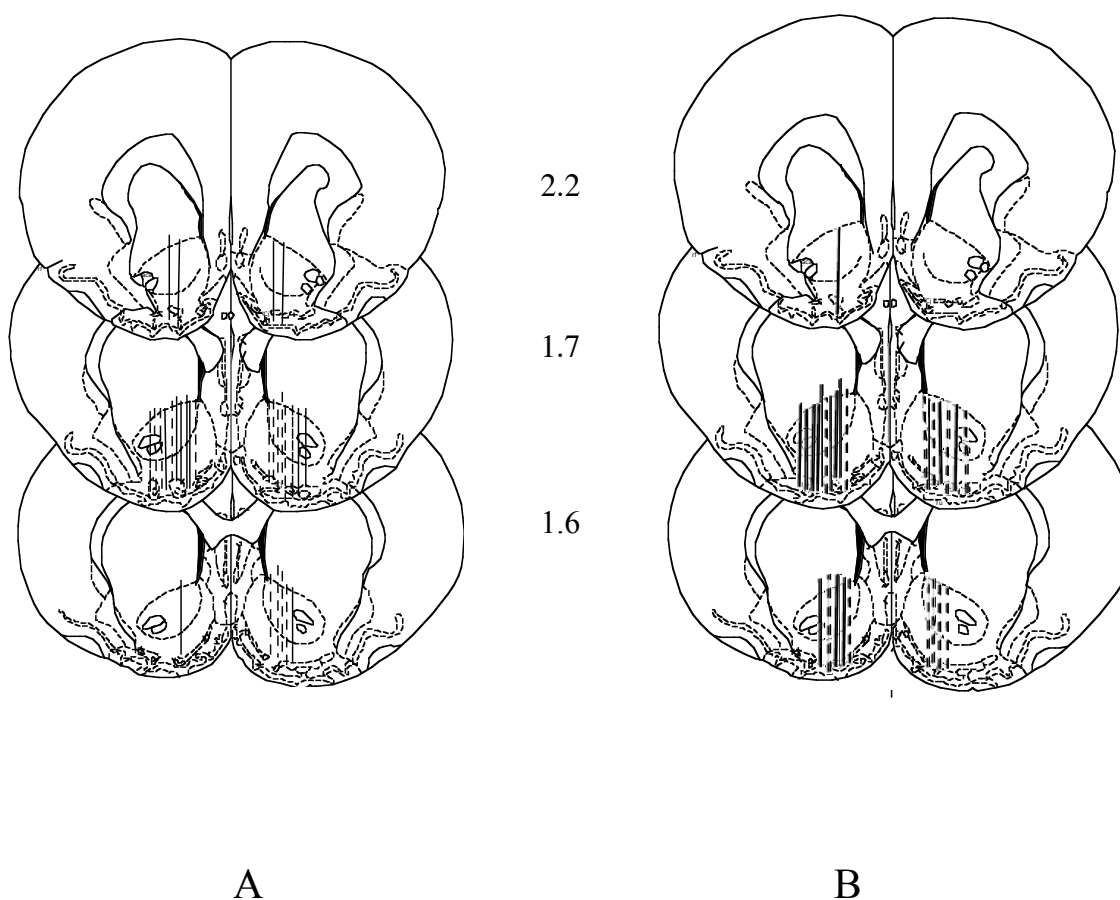


Fig. 21 . Schematic representation of active dialysis probe membrane in the NAcc of animals who underwent 'Certainty' cue-associative training with (A) Diazepam and (B) Saline pretreatment

The numbers between the two figures indicate the distance of the slice in mm anterior to bregma. The dotted lines represent the probes in animals tested with saline and the solid lines represent the probes in animals tested with cocaine on the dialysis day. The diagram was drawn with the assistance from the atlas of Paxinos and Watson (Paxinos and Watson, 1998).

Figure 22

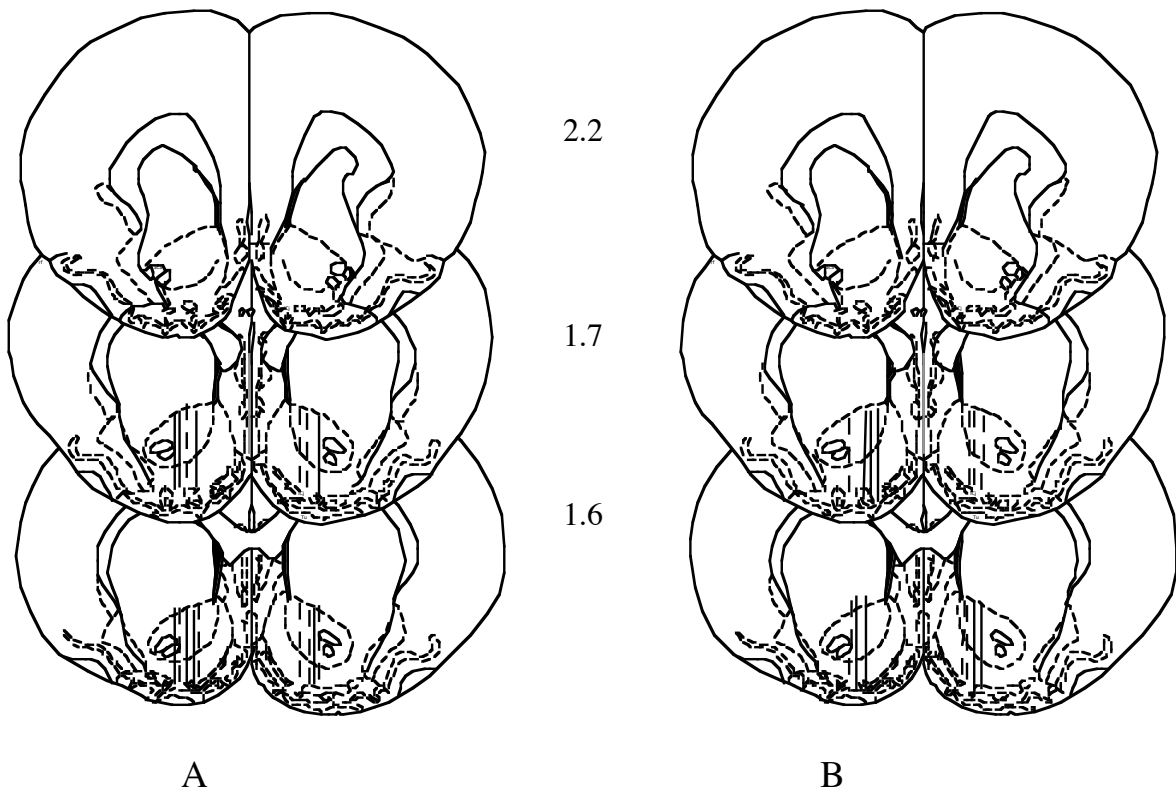


Fig. 22 . Schematic representation of active dialysis probe membrane in the NAcc of animals who underwent 'Uncertainty' cue-associative training with (A) Diazepam and (B) Saline pretreatment

The numbers between the two figures indicate the distance of the slice in mm anterior to bregma. The dotted lines represent animals tested with saline and the solid lines show animals tested with cocaine. The diagram was drawn from the atlas of Paxinos and Watson (Paxinos and Watson, 1998).

Figure 23

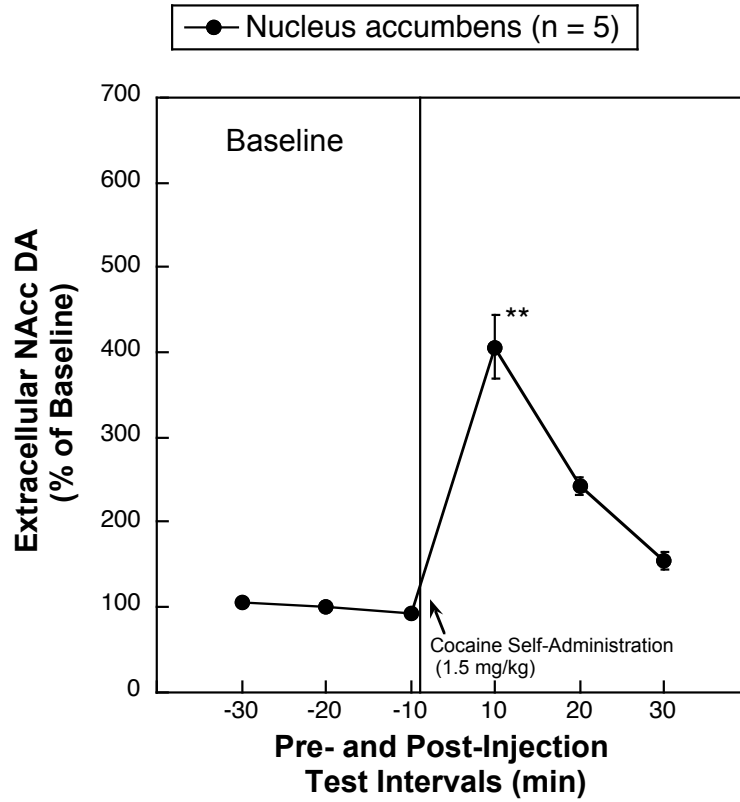


Fig. 23. Nucleus accumbens dopamine (NAcc DA) response following cocaine (1.5 mg/kg) self-administration in drug naïve animals

Following cocaine self-administration (1.5 mg/kg) there was significant increase (**p<0.01) in NAcc DA from baseline levels. The mean \pm SEM uncorrected DA nM level was 0.79 ± 0.1 .

Chapter 4: Discussion

4-1. Discussion for data from Specific Aim 1 and 2

Specific Aim 1 focused on inducing cue-induced certainty (expectation) and uncertainty with respect to cocaine reward using cue-associative training and measuring the influence of these cognitive factors on behavior (Self-administration and locomotor activity).

Specific Aim 2 determined the influence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues on NAcc DA and locomotor activity following cocaine and saline self-administration in animals that had undergone ‘Certainty’ and ‘Uncertainty’ cue-associative training.

Certainty vs. Uncertainty cue-associative training

The findings from specific aim 1 reveal a powerful influence of cue-associative training on behavioral responses. In this study, when cocaine and saline (non-rewarded) operant sessions were accompanied by consistent cue pairings, animals showed gradual acquisition of cocaine self-administration that exceeded non-reinforced responding by the last session (see Fig.1). However, even though cocaine and non-reinforced operant sessions progressed in the same alternating day pattern for all groups, the development of preferential responding for cocaine over saline was impeded when cues were not consistently presented with the same reward outcomes. Although, the preferential responding for cocaine in the ‘Certainty’ training group is not really robust, previously we have seen that the differences between cocaine and saline self-administration get exaggerated as training progresses with consistent cues for cocaine and saline (Ikegami et al., 2007). So, what we are seeing here is the beginning of this dissociation. Both types of

‘Uncertainty’ training procedures (e.g., Uncertainty 1 and Uncertainty 2) interfered with the development of preferential cocaine responding (see Fig.2A & 2B) and resulted in increased cocaine- and non-stimulated locomotor activity during self-administration sessions (see Fig.3A & 3B). Cue-associative training with the most frequent interchanges between cue/reinforcer pairings (Uncertainty 1) resulted in the highest level of cocaine- and non-stimulated (saline) locomotor activity. In ‘Uncertainty 2’ group cues associated with cocaine were used for the last 4 saline session and in three of them these animals show enhanced locomotor activity as compared to ‘Certainty’ cue-associative training group (see Fig. 3B). The enhanced locomotor activity during saline self-administration, appears to be conditioned increase in locomotor activity, due to association of the cues used during these saline sessions with cocaine. Conditioned increase in locomotor activity, to cues associated with cocaine has been previously reported in the literature (Brown and Fibiger, 1992; Fontana et al., 1993; Bell et al., 1997; Duvauchelle et al., 2000a). Taken together, the enhanced locomotor activity during cocaine and saline self-administration and lack of preferential responding for cocaine in the ‘Uncertainty’ training groups, suggests that ‘Uncertainty’ cue-associative training resulted in behavioral effects different from ‘Certainty’ cue-associative training.

NAcc DA and cocaine self-administration: Influence of ‘Uncertainty’, ‘Certainty’ and ‘Prediction Error’ cues

To the best of our knowledge there are no studies as yet assessing the influence of uncertainty with respect to drug reward on the mesolimbic dopaminergic system. In this study, animals with ‘Uncertainty’ training showed the greatest magnitude of NAcc DA responses to cocaine (see Fig.5). Our findings, showing the greatest NAcc DA response to self-administered cocaine in the presence of ‘Uncertainty’ cues corresponds to the view, that maximal DA activation occurs in the presence of maximum uncertainty (Fiorillo et al., 2003). Using Pavlovian conditioning and single unit recordings, the study

showed the most firing of midbrain dopaminergic neurons in the presence of cues equally associated with reward and non-reward. Several fMRI studies in human subjects have assessed the influence of uncertainty related to natural rewards (food, fruit juice, money) on firing of midbrain dopaminergic neurons and NAcc activity. For example, passive presentation of juice and water in a random order as compared to a definite order induced outcome uncertainty in human subjects. In this study, the authors showed that this outcome uncertainty activated the NAcc along with other regions such as the thalamus and anterior cingulate cortex. Another study using human subjects also showed sustained activation of midbrain dopaminergic neurons following cues associated with monetary reward uncertainty and this activation was maximal when the chance of getting rewarded was 50% (Dreher et al., 2006). In addition, they also showed the NAcc as the dopaminergic terminal site maximally activated in the presence of reward uncertainty. In yet another study, the NAcc showed greater activation when subjects chose larger uncertain rewards as compared to sure smaller rewards (Matthews et al., 2004).

The increase in NAcc DA following cocaine self-administration in the presence of 'Certainty' cues (cues consistently associated with cocaine) was significantly less as compared to the increase in NAcc DA in the presence of 'Uncertainty' cues (see Fig.5). This decreased response on receiving anticipated reward is consistent with single unit data from non-human primates, which report decreased activation of midbrain dopaminergic neurons following delivery of expected positive reward (Ljungberg et al., 1992; Mirenowicz and Schultz, 1994). fMRI studies in humans have also shown that the activation of the NAcc was least following occurrence of fully predicted reward (Abler et al., 2006; Spicer et al., 2007).

It must however, be pointed out that in this study we did not find any difference in NAcc DA response between animals self-administering cocaine in the presence of 'Certainty' and 'Prediction Error' cues (cues consistently associated with saline). This finding is

contrary to other studies, which have used natural rewards. For example, fMRI studies using monetary reward in human volunteers show an enhanced response in both the midbrain dopaminergic neurons and the NAcc, when rewards occur unexpectedly as compared to fully expected reward (Berns et al., 2001; McClure et al., 2003; Abler et al., 2006; Dreher et al., 2006). Similarly, single unit recordings in non-human primates, report phasic activation of dopaminergic neurons on occurrence of unexpected reward (Hollerman and Schultz, 1998). One possible explanation for this opposite finding could be that repeated pairing of cues with saline, made these cues less salient as compared to the overall context (operant chamber). Since the same context was associated with cocaine on cocaine days, it is possible that the animals started associating the context with cocaine, and had some expectation of receiving cocaine and there was no prediction error. Another reason for the lack of an enhanced response, can be attributed to differences between natural and drug rewards. It has been shown that natural and drug rewards have different effects on neural and behavioral systems (Robinson et al., 2001; Ciccocioppo et al., 2004). Thus, it is possible that ‘Certainty’ associative training and repeated cocaine self-administration resulted in neuroadaptations, that limited the ability of the NAcc to show an enhanced dopaminergic response to unexpected cocaine.

NAcc DA: Self-Administration of 0.5 mg/kg vs. 1.5 mg/kg

The above NAcc DA responses to ‘Certainty’, ‘Uncertainty’ and Prediction Error’ were seen following self-administration of 1.5 mg/kg/infusion of cocaine. During cue-associative training, animals received 0.5 mg/kg/infusion. Mesolimbic dopaminergic neurons have been reported to code for reward magnitude (Tobler et al., 2005). Tobler et al., reported greater midbrain dopaminergic neuronal firing following presentation of cues associated with rewards of greater magnitude. However, in the study of Tobler et al. in contrast to our study, distinct cues were associated with rewards of different magnitude. In our study, both ‘Certainty’ and ‘Uncertainty’ cue-associative training

groups had cues associated with 0.5 mg/kg/lever response during conditioning. To see if the dose of cocaine used during the dialysis session made a difference to NAcc DA response, animal's self-administered cocaine in the dose of 0.5 mg/kg on the dialysis day in the presence of 'Certainty', 'Prediction Error' and 'Uncertainty' cues. The data (see Fig. 9) showed that NAcc DA was significantly enhanced to the same extent in all the three cue conditions. However, it was interesting to note that the percentage increase in the NAcc DA under conditions of 'Certainty' and 'Prediction Error' cues was the same as that seen following self-administration of 1.5 mg/kg (compare Fig. 5 & Fig. 9). This fact suggests that cocaine self-administration under 'Certainty' cue-associative training limited the ability of NAcc DA to show a dose-dependent response to cocaine. This finding also strengthens our earlier explanation for the lack of enhanced response during receipt of unexpected cocaine (Prediction Error).

This brings us to the enhanced response seen in the presence of 'Uncertainty' cues, following cocaine self-administration in the dose of 1.5 mg/kg and not at 0.5 mg/kg. One possible explanation for this finding could be that the mesolimbic firing was greater after 1.5 mg/kg, due to the greater magnitude of the reward. This is not likely, as this would have resulted in an enhanced response to the same extent in all three groups because all three groups were receiving 1.5 mg/kg for the first time. Thus, the greater responding of dopaminergic neurons on account of the higher dose on the test day cannot completely explain the findings seen in our study. Another possibility is that cocaine, which acts by blocking the uptake of DA, trapped the enhanced extracellular DA better at 1.5 mg/kg as compared to 0.5 mg/kg. Thus, we saw the enhanced dopaminergic response in the 'Uncertainty' group only at 1.5 mg/kg and not at 0.5 mg/kg. It is also possible that 'Uncertainty' and 'Certainty' training induced different neuroadaptations in the animals. As a result, animals that underwent 'Uncertainty' training showed a sensitized dopaminergic response to the higher dose of cocaine as compared to animals that underwent 'Certainty' training. Finally, it is also possible that the enhanced dopaminergic

response seen at 1.5 mg/kg in the presence of ‘Uncertainty’ cues may be independent of dopaminergic firing and may have resulted from glutamate-induced DA release at the dopaminergic terminals in the NAcc.

NAcc DA and saline self-administration: Influence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues

Self-administration of non-reward (saline) in the presence of cues consistently associated with cocaine (‘Prediction Error’ cues), showed a significant depression from basal DA levels in the first 10 minutes after saline self-administration (see Fig. 6). In contrast, animals self-administering saline in the presence of cues consistently associated with saline (‘Certainty’) showed no changes from baseline in NAcc DA levels. This finding suggests that after ‘Certainty’ cue-associative training, the NAcc was still sensitive to omission of expected cocaine reward. The observed decrease in NAcc DA correlates well with findings from single unit recordings in non-human primates, which show a decrease in DA neuronal firing on omission of expected reward (Hollerman and Schultz, 1998; Waelti et al., 2001). Similarly, fMRI studies in human volunteers, also report a depression in NAcc activity following omission of expected monetary reward (Knutson et al., 2001; Abler et al., 2005). Our current findings, in case of negative prediction error, also matches up with our previous work using microdialysis and a similar but shorter conditioning paradigm (12 days) (Ikegami et al., 2007). In animals self-administering saline in the presence of saline associated cues (‘Certainty’ cues), NAcc DA remained same as baseline immediately following saline self-administration. This counters our earlier suggestion that the animals associated the chamber with cocaine, because if this was so then these animals would have also shown a decrease in NAcc DA. However, our notion may not be completely wrong as these animals did show a decrease in NAcc DA at the subsequent time point of 20 minutes (see Fig.6). Self-administration of non-reward in the presence of ‘Uncertainty’ cues resulted in a slight depression in DA levels. This

depression was not significant as compared to baseline. This partial depression in DA, suggests that these animals had some expectation of receiving cocaine, but not as strong a expectation as animals that underwent ‘Certainty’ cue-associative training.

NAcc core vs. shell: Possible roles in mediating uncertainty and prediction error

The region of the NAcc probed (see Fig.10) in this study included both core and shell, which have been shown to be involved in mediating different aspects of reward learning. The NAcc shell has been shown to be more responsive to primary rewards (food and drugs of abuse) in naïve animals (Pontieri et al., 1995; Carlezon and Wise, 1996; Tanda et al., 1997) as compared to the core, which is believed to play an important role in associative learning between environmental stimuli and primary rewards (Bassareo and Di Chiara, 1999; Parkinson et al., 1999; Ito et al., 2000). The literature however is controversial about the definitive roles of core vs. shell. A recent study suggests, that the shell might also play a role in cue-drug reward associative learning when a Pavlovian conditioning paradigm is used (Bassareo et al., 2007). Lesions of the core have been shown to decrease impulsivity and promote selection of smaller certain appetitive rewards, when animals are provided a choice between larger uncertain rewards and smaller certain rewards (Cardinal and Howes, 2005). Impulsivity and risk taking behaviors are associated with uncertainty of reward. Thus, it is possible that the core may play a more vital role in uncertainty. In contrast, since the shell is more responsive to the actual reward, it might play a more important role in prediction error. However, from our study it is difficult to make such statements as our probes trespassed both areas.

Locomotor activity and Cocaine self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues

There was significant increase in locomotor activity following cocaine self-administration in all the three groups (see Fig. 7). This is consistent with psychomotor activating effects of cocaine (Wise and Bozarth, 1987). However, the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues did not influence the increase in locomotor activity. Studies show that NAcc DA plays an important role in cocaine-induced increase in locomotor activity. For example, increase in NAcc DA in drug naïve rats following systemic administration of cocaine correlates with increase in locomotor activity (Di Chiara and Imperato, 1988; Hurd et al., 1989; Pettit and Justice, 1991). Also it has been shown that increase in locomotor activity following systemic administration of cocaine can be reversed by intracumbens injection D2 receptor antagonist sulpiride and eticlopride (Kaddis et al., 1993; Neisewander et al., 1995). In our study, increase in locomotor activity did not completely correlate with NAcc DA. For example, the increase in NAcc DA following cocaine self-administration was greatest in the presence of ‘Uncertainty’ cues but the increase in locomotor activity in these animals was not the greatest. Thus, our findings suggest a dissociation between NAcc DA increase and locomotor activity. However, other studies have also reported dissociation between NAcc DA and locomotor activity, especially after repeated cocaine administration (Kuczenski et al., 1991; Segal and Kuczenski, 1992; Ikegami et al., 2007). It is possible, that the locomotor activity was mediated by other regions such as dorsal striatum, VTA, ventral pallidum, olfactory tubercle, which are also believed to play a critical role in mediating cocaine-induced locomotor effects (Mayfield et al., 1992; Gong et al., 1996; Borgland et al., 2004; Chambers et al., 2004). Our findings, thus indicate that pathways influencing cocaine-induced locomotor activity might be different from those mediating uncertainty and prediction error associated with cocaine reward.

Locomotor activity and saline self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues

Animals self-administering saline in the presence of cues consistently associated with saline (‘Certainty’), showed a significant decrease in locomotor activity by the end of the session as compared to their locomotor activity in the first 10 minutes after presentation of cues (see Fig.8). There was however no difference in locomotor activity between animals tested in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues. Also, animals self-administering saline in the presence of cues consistently associated with cocaine (‘Prediction error’ cues) did not show an conditioned enhancement in locomotor activity. This is in contrast to studies using Pavlovian conditioning paradigms, which show an enhanced locomotor activity in contexts consistently associated with cocaine (Brown and Fibiger, 1992; Burechailo and Martin-Iverson, 1996; Duvauchelle et al., 2000a). This is possibly because of differences between Pavlovian and instrumental conditioning. In instrumental conditioning, animals have control over drug delivery and can predict the delivery of drug. In contrast, in Pavlovian conditioning paradigms the drug is experimenter-administered, and animals have no control nor can they predict the delivery of the drug. Hence, the enhanced locomotor activity in Pavlovian conditioning paradigms in cocaine-associated contexts, could be in anticipation of receiving cocaine. In our study, after self-administering saline the animals did not have any further opportunities to press the lever. Therefore, they did not anticipate receiving cocaine and thus may not have shown an enhanced locomotor response despite the presence of cocaine-associated cues. However, these animals did not show a significant decrease in locomotor activity over time, as shown by animals tested in the presence of ‘Certainty’ cues. The animals self-administering saline, in the presence of ‘Uncertainty’ cues, also did not show an enhanced response unlike that seen during cue-associative training. This again could be because of the unavailability of the lever after initial lever response.

NAcc DA: VTA neuronal firing vs. glutamatergic afferents to DA terminals

The NAcc is one of the major output sites, for dopaminergic neurons arising from the VTA (Fallon and Moore, 1978). *In vivo* voltammetry studies, have shown that firing of VTA dopaminergic neurons in response to cues (Waelti et al., 2001) corresponds with DA release in the NAcc (Phillips et al., 2003; Roitman et al., 2004). The cue-induced DA released in the NAcc, is critical for NAcc neuronal firing and reward seeking behavior. For example it has been shown, that inactivation of VTA dopaminergic neurons using GABA_B agonist baclofen, decreases NAcc DA release (Westerink et al., 1996) and NAcc neuronal firing (Yun et al., 2004). This decrease in NAcc neuronal firing also decreases reward seeking behavioral response on exposure to stimuli previously associated with reward (Nicola et al., 2005). Thus, it is reasonable to suggest that enhanced firing of VTA dopaminergic neurons resulted in increased DA release under conditions of uncertainty. Similarly, inhibition of dopaminergic firing resulted in a decrease in DA levels under conditions of negative prediction error. However, terminal DA release in the NAcc can also occur without DA cell firing through presynaptic modulation of DA terminals (Joseph et al., 2003). Endogenous excitatory amino acids glutamate and aspartate have been shown to increase NAcc DA release through presynaptic modulation of dopaminergic terminals (Youngren et al., 1993). The NAcc receives glutamatergic inputs from prefrontal cortex, amygdala and hippocampus, and these afferents are capable of releasing DA from NAcc DA terminals (Christie et al., 1987). The hippocampus, prefrontal cortex and amygdala also play important role in associating environmental cues with rewards (Isaac et al., 1989; Baxter and Murray, 2002; Milekic et al., 2006). Neuroimaging studies using fMRI in human volunteers show that the prefrontal cortex, parietal and insular cortex are activated in the presence of uncertainty (Huettel et al., 2005). It is thus possible that the enhanced DA and suppression of DA seen under conditions of uncertainty and prediction error in our study, could be because of

presynaptic influences on DA terminals from regions such as the prefrontal cortex, hippocampus and amygdala. Further work will be required to dissociate these influences.

Microdialysis vs. Single unit recordings and fMRI

In this dissertation, findings from *in vivo* microdialysis experiments have been extensively compared with findings from studies using techniques such as single unit recordings and functional magnetic resonance imaging (fMRI). Although, there is agreement between our microdialysis findings and findings from single unit recording and fMRI studies, we must keep in mind that the time resolution for these studies is vastly different. For example, microdialysis experiments report changes in extracellular dopamine in the range of minutes (10 minutes in this dissertation). In contrast, single unit recordings, voltametry, and fMRI studies report changes in the range of sub second and seconds. Thus, it is possible that microdialysis findings may not be reporting the same thing as that reported by single unit and fMRI studies and may be reflecting different aspects of dopaminergic neurotransmission.

Uncertainty and Prediction Error: Role of NAcc DA

So, what is the significance of the enhanced DA and suppressed DA levels in the presence of cocaine reward uncertainty and prediction error respectively? The NAcc is a critical site for the integration of sensory inputs from a number of limbic areas such as the prefrontal cortex, amygdala and hippocampus (O'Donnell and Grace, 1995). NAcc DA plays an important role in filtering these inputs (Horvitz, 2002; Goto and Grace, 2005) and in doing so it helps to enhance attention to salient stimuli and carry out appropriate action. In fact, a large body of literature suggests that DA plays an important role in attention (Ladurelle et al., 1997; Robbins, 1997; Puumala and Sirvio, 1998; Redgrave et al., 1999; Nieoullon, 2002; Joseph et al., 2003; Franken et al., 2005). This role of the

NAcc is best highlighted using the latent inhibition paradigm. Latent inhibition is defined as the attenuated ability to form an associative relationship between the familiar stimulus and unconditioned stimulus (Gray et al., 1995; Gray et al., 1997; Weiner and Feldon, 1997). Increase in NAcc DA either through intra-accumbens amphetamine injection or systemic amphetamine injections can disrupt latent inhibition (Solomon and Staton, 1982; Weiner et al., 1988). In other words, increased NAcc DA can help in associative learning of previously exposed stimuli. In our study the enhanced DA in the presence of uncertainty cues may serve a similar purpose of helping to associate stimuli previously associated with saline to cocaine. Similarly, a decrease in DA on omission of anticipated cocaine reward, may help to weaken established associative learning and may enhance extinction (Rescorla, 2006b).

Drug reward, Uncertainty and Prediction error: Possible implications to human drug dependence

Situations of uncertainty can by themselves be reinforcing. For example, gamblers find outcome uncertainty reinforcing and therefore they continue to gamble inspite of mounting losses (Fiorillo et al., 2003). Also, a recent fMRI study in young stimulant (cocaine, amphetamine) users (18-25yrs) has reported, that conditions of uncertainty resulted in increased striatal activations, as compared to age-matched controls that did not use drugs (Leland et al., 2006). The authors of this study concluded, that these stimulant users were reinforced by conditions of uncertainty. Cocaine-stimulated NAcc DA increase has also been associated with reinforcement (Wise and Bozarth, 1987; Koob and Weiss, 1992). Thus, it is possible that the enhanced cocaine-stimulated NAcc DA response in the presence of ‘Uncertainty’ cues may be more reinforcing. Hence, it is possible people who enjoy situations of uncertainty, may find cocaine more reinforcing under conditions of uncertainty, as compared to those who do not enjoy such situations. The interaction of uncertainty and cocaine is not unlikely as people who initiate drug

taking maybe facing situations with uncertain outcomes due to various conflict situations in their lives.

Enhanced mesolimbic DA in animals has been associated with sensitization of reward pathways and this is suggested to result in increased motivation and persistence of behaviors (Vezina et al., 2002). It is therefore possible, that when people experience cocaine under conditions of uncertainty, they may be more vulnerable to develop drug dependence. Retrospective surveys in individuals with substance abuse problems have found that impulsivity and risk taking behavior are common traits among these people (Dawe and Loxton, 2004). Impulsivity and risk taking behaviors are often exposed, when individuals are facing situations of uncertainty. It is possible, that people who are impulsive show a greater response to cocaine, and therefore may be more vulnerable to develop drug dependence?

The other question is, whether the enhanced DA is associated with more pleasure? In a study done in cocaine dependent patients, it was observed that enhanced activation of the NAcc was indeed associated with enhanced pleasure (Breiter et al., 1997). Thus, it is possible that cocaine under conditions of uncertainty may be more pleasurable. In the future, studies assessing the subjective experience of cocaine in cocaine-dependent subjects, may also have to take into account influence of cognitive factors such as uncertainty on these subjective experiences.

The decrease in NAcc DA on omission of expected cocaine reward is significant (Prediction Error) and it is possible that this depression may help to weaken cue-cocaine association. This weakening of cue-cocaine association may enhance extinction learning. Extinction is an inhibitory form of learning and helps to suppress drug-seeking behavior. If induction of prediction error helps extinction learning then it may be possible to develop it as behavioral therapy to prevent drug-use relapse in recovering addicts. In

recovering addicts, expectations of receiving cocaine can be built up with the help of environments associated with cocaine and then prediction error can be induced, by administering saline or placebo. This may help to extinguish cocaine-seeking behavior and help in relapse prevention. However, further work is essential to conclusively prove that extinction is faster and more effective using this prediction error strategy.

Conclusion

In summary, the findings of this study show that cocaine self-administration under conditions of uncertainty, leads to enhanced NAcc DA response. Also, suppression in NAcc DA is seen on omission of expected cocaine reward. Future work must try and focus on the relevance of these findings in terms of synaptic plasticity at the level of the medium spiny neurons. In addition, we must also see if it is possible to disrupt these learned NAcc DA responses by blocking acquisition of cue-cocaine associative learning.

4-2. Discussion for Specific Aim 3

Findings from Specific Aim 2 showed, that in the presence of ‘Uncertainty’ cues (cues equally associated with cocaine and saline), NAcc DA response following cocaine self-administration was significantly enhanced as compared to animals self-administering cocaine in the presence of cues consistently associated with cocaine (‘Certainty’ cues). Also, following self-administration of saline (non-reward) in the presence of cues consistently associated with cocaine (‘Prediction Error’ cues), there was a significant depression from baseline in NAcc DA. The objective of this specific aim was to see if diazepam pretreatment (0.25 mg/kg) during cue-associative training, disrupted NAcc DA responses to cocaine and saline self-administration under conditions of uncertainty and prediction error respectively.

We found, that diazepam pretreatment during cue-associative training, disrupted the differential cocaine–stimulated NAcc DA response seen in the presence of ‘Uncertainty’ and ‘Certainty’ cues. Also, following saline self-administration, diazepam pretreated animals did not show significant depression in NAcc DA in the presence of cocaine associated cues (‘Prediction Error’ cues). In addition, findings from the odor recognition task showed that diazepam did not affect acquisition of olfactory memory. These findings suggest, that diazepam pretreatment during cue-associative training selectively disrupted acquisition of associative learning between cues and cocaine without affecting olfactory memory.

Cue-associative training: Diazepam vs. saline pretreatment

During cue-associative training, animals self-administered cocaine and saline in the presence of olfactory and visual cues. Since self-administration was involved, it was important that animals were not sedated or motor-impaired by diazepam pretreatment

during cue-associative training. A previous study in our lab showed, that diazepam in the dose of 0.25 mg/kg did not impair self-administration or motor activity of the animals (Maier et al, under revision). Hence, we decided to use an intravenous dose of diazepam of 0.25 mg/kg intravenously. Following diazepam and saline (control) pretreatment, animals underwent two types of cue-associative training, 'Certainty' and 'Uncertainty'. During 'Certainty' cue-associative training, distinct olfactory and visual cues were used for cocaine and saline (i.e. one visual and olfactory cue for cocaine and another distinct set of visual and olfactory cues for saline). During 'Uncertainty' cue-associative training, cues were equally paired with cocaine and saline. Diazepam pretreated animals undergoing 'Certainty' training showed significantly greater cocaine self-administration on days 9 and 13 as compared to saline pretreated counterparts (see Fig.11A). This enhanced cocaine self-administration following diazepam pretreatment, is consistent with findings from the previous study done in our laboratory (Maier et al., under revision). This enhanced cocaine self-administration could be due to the anxiolytic effects of diazepam. Along with its rewarding effects, cocaine has also been reported to produce anxiogenic effects in animals and humans (Smith, 1986; Louie et al., 1989; Ettenberg, 2004). Diazepam pretreatment has been shown to help overcome the anxiogenic effects of cocaine (Ettenberg and Geist, 1991; Paine et al., 2002). However, diazepam pretreatment did not influence cocaine self-administration in animals undergoing 'Uncertainty' cue-associative training (see Fig.11B). Also, diazepam pretreatment did not influence saline self-administration, in both 'Certainty' and 'Uncertainty' cue-associative training groups (see Fig.12A & 12B).

In addition, diazepam pretreatment did not influence cocaine-induced increase in locomotor activity, in both 'Certainty' and 'Uncertainty' cue-associative training groups (see Fig.13). During saline self-administration, saline pretreated animals undergoing 'Certainty' training showed significantly greater locomotor activity on days 2,4,6 and 16 as compared to their diazepam pretreated counterparts (see Fig. 14). This decreased

locomotor activity was also seen in diazepam pretreated animals undergoing ‘Uncertainty’ cue-associative training but it was not statistically significant in these animals. The decreased locomotor activity in diazepam pretreated animals suggests that there was some motor impairment (muscle relaxation) in these animals. However, this motor-impairment was not significant enough to impair self-administration behavior nor was it significant enough to impair cocaine-induced increase in locomotor activity. Although, diazepam pretreated animals did show lower locomotor activity levels during cocaine-self-administration, it was not statistically significant as mentioned earlier (see Fig.13). In specific aim 1, we had seen enhanced locomotor activity during saline self-administration in animals undergoing ‘Uncertainty’ cue-associative training. In this specific aim, we could not compare locomotor activity in saline pretreated animals undergoing ‘Certainty’ and ‘Uncertainty’ training due to differences in their basal activity. However, it must be pointed out that saline pretreated animals undergoing ‘Uncertainty’ training, did show a gradual increase in locomotor activity over time and their locomotor activity during training sessions on days 10, 12, 14 and 16 was greater than their locomotor activity on days 2 and 4.

NAcc DA and cocaine self-administration: Influence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in diazepam and saline pretreated groups

Saline pretreated animals (controls) showed a significant increase in NAcc DA following cocaine self-administration in the presence of ‘Uncertainty’ cues as compared to ‘Certainty’ and ‘Prediction Error’ cues (see Fig. 15A). Thus, we were able to repeat our findings of specific aim 2. This enhancement under conditions of cue-induced uncertainty is in keeping with several other studies in the literature, which show enhanced mesolimbic dopaminergic activation under uncertainty conditions (Fiorillo et al., 2003; Matthews et al., 2004; Fiorillo et al., 2005; Dreher et al., 2006). In diazepam pretreated animals, cocaine self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and

‘Prediction Error’ cues resulted in a significant enhancement in NAcc DA over baseline (see Fig. 15B). However, there was no significant difference between ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ groups. Thus, diazepam pretreatment during cue-associative training blocked the differential cocaine–stimulated NAcc DA response to ‘Uncertainty’, ‘Certainty’ and ‘Prediction Error’ cues. The direct effect of diazepam on NAcc DA responses is ruled out, because no diazepam was given on the dialysis day (test day) and dialysis session was conducted 24 hours after the last diazepam pretreatment. So, diazepam most likely blocked acquisition of cue-associative learning, and thereby disrupted the influence of cue-induced certainty, uncertainty and prediction error on NAcc DA response to cocaine self-administration.

NAcc DA and cocaine self-administration: Diazepam pretreatment attenuates tolerance seen following anticipated cocaine self-administration

Another significant finding was that NAcc DA response following cocaine self-administration in the presence of ‘Certainty’ cues was significantly greater in diazepam-pretreated animals as compared to saline pretreated animals (see Fig. 15B). The decreased percentage increase in saline pretreated animals (controls) in the presence of cocaine-associated cues can be accounted for by the expectation of receiving cocaine. Several studies report decreased activation of mesolimbic dopaminergic system on receipt of expected reward (Ljungberg et al., 1992; Spicer et al., 2007). The enhanced cocaine-stimulated NAcc DA response in diazepam pretreated animals is possibly due to the disruptive effect of diazepam on cue-cocaine associative learning. The cocaine-stimulated percentage increase of about 280% in NAcc DA in the presence ‘Certainty’ cues in saline pretreated animals was significantly less than that seen following self-administration of cocaine (1.5 mg/kg) in drug-naïve animals (approximately 400%) [see Fig. 23 and (D'Souza and Duvauchelle, 2006)]. Although, we cannot directly compare these studies, it appears that saline pretreated ‘Certainty’ cue-associative training animals showed

tolerance to the neurochemical effects of cocaine. One study, has suggested that tolerance to the effects of cocaine is more likely to develop following presentation of discriminative stimuli (Goeders et al., 1997). Also, Pavlovian conditioning studies have shown that tolerance to the effects of the drug occurs in the presence of cues consistently associated with the drug rather than cues not associated with the drug. For example, tolerance to the hyperthermic and analgesic effects of opioids occurs in the presence of cues consistently associated with morphine (Siegel, 1976, 1978). Tolerance is also known to occur more efficiently during self-administration. This fact has been elegantly shown in experiments using self-administration and yoked controls. For example, cocaine-induced mortality to lethal doses of cocaine was significantly lower in self-administering animals as compared to yoked controls, even-though both groups of animals got the same amount of the drug (Dworkin et al., 1995). During self-administration animals can anticipate the delivery of the drug. This anticipation brings about compensatory changes and these compensatory changes bring about tolerance to the effect of the drug. Thus, it appears that diazepam pretreatment is not only blocking cue-associative training, but in the process also attenuating the development of tolerance. Neurochemical tolerance to repeated cocaine intake, is suggested to be caused by either decrease in dopamine synthesis (Trulson et al., 1987; Trulson and Ulissey, 1987), depletion in dopamine stores (Roy et al., 1978) or alterations of release and uptake processes (Hurd et al., 1989). Thus, the anticipatory responses possibly influence one of these processes. The blockade of cue-cocaine associative learning in diazepam pretreated animals, possibly blocked the development of anticipatory responses in these animals as compared to saline pretreated animals. It is also possible, that diazepam pretreatment may have had a direct effect on the above-mentioned processes involved in neurochemical tolerance to cocaine.

NAcc DA and saline self-administration: Influence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues in diazepam and saline pretreated groups

Saline pretreated animals (controls), showed a significant decrease in NAcc DA from baseline following saline self-administration in the presence of ‘Prediction Error’ cues (cocaine-associated cues) (see Fig. 16A). Also, saline self-administration resulted in no decrease from baseline in NAcc DA in the presence of ‘Certainty’ cues. ‘Uncertainty’ cues caused a slight but not significant decrease in NAcc DA from baseline following saline self-administration in saline pretreated animals. All these findings are similar to our findings in specific aim 2. The decrease in NAcc DA on omission of expected reward has been reported by several other studies (Hollerman and Schultz, 1998; Abler et al., 2006; Ikegami et al., 2007; Spicer et al., 2007). In diazepam pretreated animals, saline self-administration resulted in slight decrease in NAcc DA from baseline in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues. However, this decrease in DA was not statistically significant. Thus, diazepam pretreatment during cue-associative training, blocked the significant decrease in NAcc DA seen following saline self-administration in the presence ‘Prediction Error’ cues.

Locomotor activity following cocaine and saline self-administration in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues: Diazepam vs. saline pretreatment

Cocaine self-administration enhanced locomotor activity in both diazepam and saline pretreated groups in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues (see Fig.17A & 17B). No significant differences were observed between the saline and diazepam pretreated groups under the different cue conditions. Thus, diazepam pretreatment during cue-associative training did not influence locomotor response to cocaine during dialysis. Thus, we once again found a dissociation between NAcc DA and

locomotor activity (see findings from specific aim 2). This dissociation has been reported by other studies (Kuczenski et al., 1991; Segal and Kuczenski, 1992; Ikegami et al., 2007). This dissociation, suggests that following repeated cocaine exposure, psychomotor responses to cocaine may be mediated by other regions such as the VTA, dorsal striatum, ventral pallidum and/or olfactory tubercle (Mayfield et al., 1992; Gong et al., 1996; Borgland et al., 2004; Chambers et al., 2004). Similarly, following saline self-administration no significant differences in locomotor activity were observed between saline and diazepam pretreated groups in the presence of ‘Certainty’, ‘Uncertainty’ and ‘Prediction Error’ cues (see Fig. 18A & 18B).

Influence of diazepam pretreatment on olfactory memory

It is possible, that diazepam disrupted cue-induced learned responses by blocking acquisition of olfactory memory. Hence, we used the odor recognition task (see methods) to see if diazepam pretreatment (0.25 mg/kg) blocked acquisition of olfactory memory (Spinetta et al., 2006). Diazepam pretreatment did not affect habituation, and the habituation was similar to saline pretreated animals (see Fig. 19). The results of the odor recognition task the following day, were similar in both diazepam and saline pretreated animals suggesting that diazepam pretreatment did not affect the acquisition of olfactory memory (see Fig. 20). Thus, we can conclude that diazepam pretreatment selectively disrupted the cue-induced learning with respect to cocaine reward, without affecting olfactory memory.

Disruption of cocaine-associative learning by diazepam : Possible mechanisms

Diazepam in the dose of 0.25 mg/kg does not influence cocaine-stimulated NAcc DA levels (Maier et al., 2007 under revision). Diazepam has been shown to reduce NAcc DA release at higher doses (5, 10, 20 mg/kg) but not at 1 mg/kg (Invernizzi et al., 1991). So,

the mechanism of diazepam-induced disruption of cocaine-associative learning in this study, does not appear to be mediated by inhibition of DA release. One possible mechanism by which diazepam can block the acquisition of cocaine-associative learning is by its inhibitory action on cocaine-induced LTP (Liu et al., 2005). In this paper, Liu et al. showed that repeated cocaine administration induced LTP in VTA dopaminergic neurons. They also showed that this induction was due to a decrease in GABAergic tone and enhancing GABA_A receptor activation via diazepam blocked this cocaine-induced LTP. LTP is a form of synaptic plasticity, and one study has shown that it plays an important role in associative learning involving aversive stimuli (Rumpel et al., 2005). Thus, it is possible that by enhancing GABAergic tone through diazepam pretreatment during cue-associative training, we were able to block cocaine-induced LTP and thus disrupt cocaine-associative learning. Another possible mechanism is through diazepam-mediated inhibition of D1 receptor signaling. An *in vitro* study, using synaptosomal membranes has shown that diazepam inhibits brain adenylate cyclase (Dan'ura et al., 1988) via benzodiazepine receptor coupled inhibitory GTP-binding protein (Gi) (Kurokawa et al., 1988). D1 receptor stimulation, activates adenylate cyclase, which leads to cAMP formation (Missale et al., 1998). The D1-cAMP-PKA pathway plays an important role in drug-associative learning (Nestler, 2001) and has been shown to be upregulated by repeated cocaine administration (Henry and White, 1995; Neisewander et al., 1996; Nestler, 2001). D1 receptor antagonists block cocaine-induced place preference (Cervo and Samanin, 1995). Thus, it is possible that by blocking D1 receptor activation, diazepam may be blocking cue-cocaine associative learning. Also, diazepam pretreatment (5 mg/kg *i.p.*) 10 minutes prior to amphetamine (5 mg/kg *i.p.*) has been shown to block *c-fos* expression in the rat striatum (Niles et al., 1997). *C-fos* is a downstream effector of D1 receptor activation and its expression is increased on exposure to cocaine-associative environments (Brown et al., 1992; Miller and Marshall, 2005b). Thus, blocking *c-fos* expression may be yet another possible mechanism through which diazepam blocks cue-cocaine associative learning.

Diazepam and Cocaine: Does diazepam pretreatment enhance cocaine dependence or blocks cocaine-associative learning?

In animals undergoing 'Certainty' training, diazepam pretreatment during cue-associative training resulted in an enhanced DA response to cocaine as compared to saline pretreatment. In fact the response was similar to that seen in saline pretreated animals undergoing 'Uncertainty' training. So, does this enhanced response seen in diazepam pretreated animals make animals more prone to drug dependence? We believe that the enhanced cocaine-stimulated DA response seen in diazepam pretreated animals reflects a lack of anticipation of cocaine and lack of cue-cocaine associative learning. So, can we differentiate this lack of learning from animals that underwent 'Certainty' and 'Uncertainty' training under saline pretreatment. One possible way is to put these animals through extinction training. Extinction is an inhibitory form of learning, which suppresses cocaine-seeking behavior. So, during extinction we can let both diazepam and saline pretreated groups self-administer saline everyday in the absence of cues. This will attenuate expectations of cocaine following lever responses. After lever response behavior undergoes extinction, animals can be tested in the presence of cues previously associated with cocaine. Animals that had learned to associate the cues with cocaine will show reinstatement of lever response behavior, while animals that did not associate the cues with cocaine will not show reinstatement of lever response behavior. We predict that diazepam pretreated animals will not show reinstatement of lever response behavior, as we propose that diazepam pretreatment disrupted the learning between cues and cocaine.

Conclusion

In summary, the findings of this study show that diazepam pretreatment disrupts the differential cocaine-stimulated NAcc DA response seen in the presence of 'Uncertainty' and 'Certainty' cues. Also, it disrupts the significant decrease in NAcc DA on saline self-

administration in the presence of cocaine-associated cues ('Prediction Error' cues). Thus, diazepam pretreatment during cue-associative training disrupts cue-cocaine associative learning. Also, it appears that diazepam pretreatment prevents the development of neurochemical tolerance, which is seen in the saline pretreated animals undergoing 'Certainty' cue-associative training. Future investigations must focus on the synaptic and molecular mechanisms mediating diazepam-induced disruption of cocaine-associative learning and cocaine tolerance. Identification of these mechanisms will help us develop effective medications to counter cue-induced drug-use relapse.

Chapter 5: Future Directions

The main findings of this dissertation can be summarized as follows:

- 1) Animals undergoing ‘Uncertainty’ cue-associative training showed enhanced locomotor activity during saline self-administration and also do not acquire preferential responding for cocaine.
- 2) ‘Uncertainty’ cues (cues equally associated with cocaine and saline) enhanced NAcc DA response following cocaine self-administration as compared to ‘Certainty’ cues (cues consistently associated with cocaine).
- 3) Self-administration of saline in the presence of cues associated with cocaine (‘Prediction Error’ cues) resulted in a significant depression in NAcc DA below baseline.
- 4) Diazepam pretreatment during cue-associative training abolished the differential cocaine-stimulated NAcc DA response in the presence of ‘Uncertainty’ and ‘Certainty’ cues.
- 5) Diazepam pretreatment during cue-associative training also attenuated the significant decrease in NAcc DA following saline self-administration in the presence of ‘Prediction Error’ cues.
- 6) Diazepam pretreatment during cue-associative training also attenuated the development of neurochemical tolerance to cocaine self-administration in animals undergoing ‘Certainty’ cue-associative training.

Thus, future work must make an attempt to identify neuroadaptations, neural circuitry and other neural substrates responsible for these findings. For example, the source of the enhanced NAcc DA response following cocaine self-administration in the presence of ‘Uncertainty’ cues. As mentioned in the discussion, the NAcc is a terminal area for the dopaminergic neurons arising in the VTA. Thus, a logical assumption is that the enhanced NAcc DA response is due to enhanced firing of VTA neurons. However, NAcc

DA can also be released through glutamatergic afferents from the PFC, which is also known to play a role in reward uncertainty. Thus, it is essential to identify the relative contribution of the VTA and PFC in the enhanced NAcc DAergic output. One way in which this can be done is by inactivation of the VTA using localized injections of lidocaine prior to testing. Another way would be to measure glutamate levels along with DA levels, to see if there is an enhancement of glutamate along with DA. This will not entirely suggest involvement of PFC as glutamatergic afferents can come from amygdala and hippocampus, which are also involved in cue-associative learning. It will also be interesting to explore the responses of DA in the dorsal striatum under conditions of 'Certainty' and 'Uncertainty'. It is possible, that dorsal striatal DA may be enhanced more under conditions of 'Certainty' than 'Uncertainty'. This prediction is based on the evidence that suggests that the dorsal striatum is more involved in habitual responding (Vanderschuren et al., 2005). Finally, as mentioned in the discussion, the roles of core and shell in mediating cocaine-induced responses under conditions of 'Certainty' and 'Uncertainty' need to be further explored.

Pretreatment with diazepam during 'Certainty' cue-associative training indicated that animals did not learn the relationship between cues and cocaine, and thus showed response more like animals that underwent 'Uncertainty' cue-associative training. The question is, what is site of action of diazepam? Is it at the level of the VTA or some other neural target? Localized injections of diazepam in the VTA, is one way to identify the site of action. Findings of this study highlight the role of GABA in cocaine-associative learning. It is suggested, that a decrease in GABAergic tone may play an important role in formation of cocaine-associative memories (Liu et al., 2005). Thus, we can use our model to evaluate other GABAergic agents such as vigabatrin (GABA transaminase inhibitor) and tiagabine (GABA uptake inhibitor). These agents will increase the GABAergic tone, and this will give us an idea about the relative importance of GABA_A activation vs. generalized increase in GABAergic tone in cocaine-associative learning.

Since, we propose here that a decrease in GABAergic tone is responsible for cocaine-associative learning, it needs to be seen if injections of GABA_A antagonists actually enhance cocaine-associative learning. Our results also suggest that diazepam may attenuate the development of neurochemical tolerance to cocaine. Further investigation will be needed to see if this is a benzodiazepine specific effect or a general GABAergic effect.

Chapter 6: Conclusion

Our findings show that behavioral and neurochemical responses to cocaine are not merely pharmacological responses but are also influenced by cognitive factors such as ‘Uncertainty’ and ‘Prediction Error’. So, what is the significance of these findings for human drug-dependence and treatment? Human subjects may face uncertainty due to various conflict situations in their lives and the interaction of cocaine and outcome uncertainty is not an impossible scenario. It is possible, that people experiencing uncertainty may have different subjective experiences on taking cocaine as compared to people who are not facing similar situations. It is also possible, that the interaction of uncertainty and cocaine may lead to neuroadaptations in brain circuits that may help in the transition from drug-use to drug-dependence. Therefore, it is essential that future drug-dependence studies in human subjects look specifically at the influence of uncertainty on response to drugs like cocaine using subjective and objective measures. Also, the decrease in DA on omission of expected cocaine reward may weaken cue-cocaine associations, and inducing prediction errors could be an effective way to induce extinction, in patients of cocaine dependence. Finally, diazepam may be an effective agent to disrupt cue-cocaine associative learning during acquisition, and future studies must look at its role in disrupting already formed cue-cocaine associations.

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